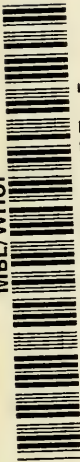






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# THE VIRUSES

Volume 2

PLANT AND BACTERIAL VIRUSES



# THE VIRUSES

Biochemical, Biological, and Biophysical Properties

*Edited by*

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Volume 2

PLANT AND BACTERIAL VIRUSES



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## Chapter I

### The Process of Infection and Virus Synthesis with Tobacco Mosaic Virus and Other Plant Viruses

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#### I. INTRODUCTION

During the 60 years since viruses were first discovered in plants, an enormous literature has accumulated. The rate of accumulation was slow at first. Then, in the middle 1930's, the rate took a sharp upturn as the result

of the monumental discovery that plant viruses are nucleoproteins. Except for the war years, the rate of published paper production has continued to rise, with no abatement in sight.

The welter of plant virus literature can be classified into three main compartments. The largest compartment contains a wealth of descriptive information on the great variety of diseases which viruses produce in plants, the mode of transfer of viruses from plant to plant, kinds of symptoms induced in the hosts, etc. A second compartment contains a truly impressive knowledge of the chemical composition and physical construction of plant viruses as they are observed in an extracellular condition. The third compartment contains our current, but insufficient knowledge of the mechanisms involved when a virus interacts with a plant cell to set in motion the phenomenon of virus self-replication. Much of the experimental information has come from the study of tobacco mosaic virus (TMV). Compared to the others, the third compartment is just beginning to fill. Enough is known, however, to trace the rough outlines of virus development within living plant cells, and sketching this picture will be the objective of this chapter.

Since it has not yet been possible to separate plant cells in a way that would permit investigation of virus activity within single cells, as in the case of bacteriophage and animal viruses, all of our information rests on the analysis of virus infection of plant organs. Since the leaf has served as the principal organ for experimentation, it is appropriate to commence this discussion by considering the organization of a typical leaf before proceeding toward analyzing the effect of virus infection on leaf cells.

## GENERAL FEATURES OF THE HOST-VIRUS SYSTEM

### *A. Organization of Plant Leaves*

Seen in cross section, a leaf is a multilayered structure. Both the upper and lower surfaces of the leaf are covered by a single layer of epidermal cells. Directly beneath the upper epidermis layer is found another single layer of closely packed, columnar cells known as the palisade parenchyma layer. These elongated cells have their greatest dimension running at right angles to the epidermal layer. Beneath the palisade layer are found a few layers of loosely packed cells known as the spongy mesophyll. These cells are more or less circular in outline and, like the palisade layer, the spongy cells are packed with green chloroplasts. The lowermost layer of the spongy mesophyll is bounded by another single layer of cells, the lower epidermis. The two epidermal layers are virtually indistinguishable from each other, but are sharply set apart from the palisade and spongy layers by the fact that they contain few chloroplasts and appear almost colorless.

The leaf is further characterized by the presence of small pores, or stomata, appearing in the epidermal layers. The stomata permit the entrance and exit of gaseous materials from the palisade and spongy layers of the leaf. Furthermore, in the two species of tobacco extensively used in TMV studies, the upper and lower surface of the leaves are densely covered by leaf hairs. Leaf hairs are extensions of epidermal cells, and in the case of *Nicotiana glutinosa* and *N. tabacum*, are of four different kinds, which vary from being composed of only 1 or 2 living cells to as many as 6 to 7 cells, arranged in a row and capped by a group of small cells at the tip of which there is secreted a sticky substance that adheres to fingers when they come in contact with tobacco plants.

Looking down on the upper surface of a tobacco leaf, one would expect to see about  $10^4$  epidermal cells per mm.<sup>2</sup> Roughly one-third of these cells would have leaf hairs protruding from their surface. Each epidermal cell, in turn, covers between 4 and 5 palisade cells. Coursing through the interior of the leaf are still other types of cells which comprise the veins and vein endings. The average tobacco leaf contains about  $10^8$  cells.

A conspicuous feature which sets the plant cell apart from the animal and bacterial cell is the presence of a cellulose cell wall. From all available evidence, the cellulose wall is an impenetrable barrier to the passage of even the smallest protein molecules (Chibnall, 1939; Wildman and Cohen, 1955). However, where leaf cells abut on each other, the cellulose wall is pierced by tiny openings through which strands of protoplasm, known as plasmadesmata, connect the protoplasm of one cell to another. Thus, we can look at the leaf, on the one hand, as being completely surrounded by a total cellulose barrier, while, on the other hand, the protoplasm can be viewed as a continuum. Consequently, once passage through the cellulose barrier is gained by a foreign object such as a virus, the gate is opened for its potential passage through every cell of the leaf.

Another feature that contrasts a leaf with animal organs and bacterial populations is the fact that most of the visible growth of a leaf is by the process of cellular elongation and expansion. Origin by mitosis of the new cells which will develop into a leaf occurs very early in development and mainly before the leaf becomes visible to the naked eye.

This growth behavior results in the peripheral distribution of the protoplasm in the leaf cells. The thin layer of protoplasm enclosed in the cell wall in a fully grown cell may be less than 10 microns thick, and surrounds a central volume known as the vacuole, the latter accounting for 80-90 % of the total cell volume. As nearly as can be told, the vacuole is devoid of protein and visible structure, and is thought not to participate in virus reproduction.

### *B. Exclusion of Some Plant Viruses from Dividing Cells and Embryonic Tissues*

One of the striking features of TMV infection is the apparent exclusion of the virus from meristems and embryonic tissues. Even though a plant may be so thoroughly infected with the virus that symptoms are apparent in the flower petals, some unknown process operates to exclude the virus from the seed which will develop from the flower. To the writer's knowledge, there is no confirmed report that TMV infection is ever transmitted through the seeds. Equally interesting is the apparent exclusion of the virus from the dividing cells of the meristem of plants (Bennett, 1940). However, once mitoses are completed and the growth by expansion has commenced, the virus can readily be demonstrated to be present, although the amount of virus synthesized in young versus old cells is not strikingly different, according to the experience of this laboratory. Exclusion from embryonic tissue is not a general property of plant viruses, for some, like lettuce mosaic, are transmitted with high frequency via seed.

The activity of TMV also seems to be indifferent to the internal state of the protoplasm. This indifference is illustrated by the fact that the virus seems to multiply during any stage of leaf development after mitoses are completed. As the cells in the leaf expand, there is a rapid increase in protein (Dorner *et al.*, 1958) and the leaf may be said to be in a stage of net protein synthesis. The rise in protein ceases when the leaf stops expanding. Thereafter, as the leaf ages, there is a steady decline in protein, or a condition of net protein loss. However, the virus does not distinguish between these phases of leaf development, for it will infect and multiply in the leaf practically up to the time the leaf withers and falls off the plant.

### *C. Strains of Plant Viruses and Host Reactions*

Like other viruses, such as the phage T-even and T-odd series, TMV and many other plant viruses consist of a complex of many different strains, each being primarily distinguished from other strains by the specific symptoms produced in a host plant. Indeed, with TMV, it has been said that there are as many strains as investigators who have sought to identify them. However, for the purposes of our discussion, there are only two strains that have been subjected to investigation that falls within the compass of this article. One of these is the common strain, or U1, as designated in this laboratory, and the other is U2 (Siegel and Wildman, 1954). When a *N. tabacum* var. Turkish Samsun plant is infected with U1, a characteristic mottling symptom develops on leaves above the leaf which serves to initiate the infection. Similarly, U2 also produces a symptom in this kind of tobacco, but the symptom is less noticeable than U1. In other varieties of *N. tabacum* plants, U1 may



give the same type of symptom as in Turkish Samsun, but U2, more often than not, will not spread from the inoculated leaf to produce a symptom in other leaves; instead, it will produce necrotic spots on the inoculated leaf. Symptom development arises from the *systemic* invasion of all parts of the plant, in contrast to the *local lesion* response, which reveals the presence of the virus when cells become necrotic and die as the result of virus infection.

### III. BEHAVIOR OF PLANT VIRUSES IN A LOCAL LESION HOST

#### *A. Local Lesion Assay for Infectivity*

When TMV is rubbed over the surface of *N. glutinosa* leaves, discrete necrotic spots will appear on the rubbed leaf in about 2 days. Production of local lesions can be induced by TMV in other hosts, such as pinto bean, *Datura*, etc. Local lesions can also be induced by other plant viruses as well. In 1929, Holmes made the important observation that the number of lesions induced by TMV is a function of the concentration of the virus applied. His discovery thereby gave rise to the local lesion assay for measuring plant virus infectivity. Mathematical analysis of the infectivity-dilution curve by numerous workers (Bald, 1937, 1950; Lauffer and Price, 1945; Kleczkowski, 1950; Youden *et al.*, 1935) has led to the general acceptance of the concept that lesions can arise from infection of single cells by single virus particles.

Estimating plant virus activity is not as straightforward as might be desired, because the test plants vary so markedly in sensitivity in terms of absolute numbers of lesions arising from the same concentration of virus. Sensitivity changes can be very great on a day-to-day basis. Smaller changes in sensitivity appear on an hourly basis (Matthews, 1953), as well as on a leaf level and plant-to-plant basis. Efforts to level out changing sensitivity by more precise environmental control suggest that nothing less than a Phytotron would produce plants with a significant reduction in sensitivity differences.

While sensitivity differences represent a hurdle of no mean proportions, it does appear that the relative number of lesions produced as a function of virus concentration is a constant within limits of experimental error. With sufficient repetition and adequate randomization, virus dilution curves, such as that shown in Fig. 1, can be produced. Furthermore, the experimental points fit rather well a theoretical curve calculated from the Poisson distribution on the expectation that each infection would arise from a single infectious unit of virus activity. The curve is linear over a fairly wide range of virus concentrations, but bends toward the abscissa as virus concentration is increased. Since the surface of the leaf contains many millions of cells, and the curve bends when only hundreds, or, at the most, thousands of lesions

arise, the disparity between the huge number of cells present on the surface and the small number that become infected and produce a lesion requires another factor to be taken into account, namely, the concept of the susceptible site. It is the difficulty in making susceptible sites that is considered to limit the extent of initial infection, rather than differences inherent in the capacity of cells to support virus infection.

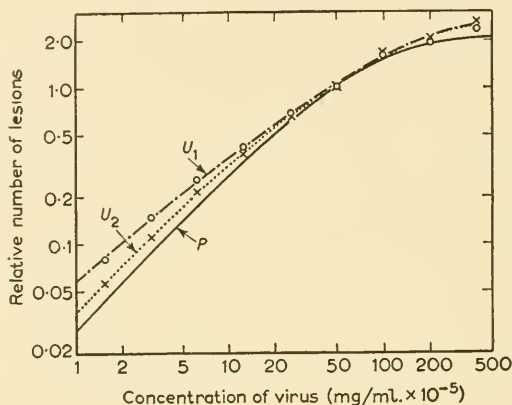


FIG. 1. Relationship between number of lesions and concentration of two strains of TMV. *P* is a theoretical curve for the expression,  $Y/N = 1 - e^{-m}$ , where  $Y/N$  = relative number of lesions, and  $m$  = average number of infectious units per susceptible site,  $m$  ranging from 0.28 to 5.6 units per site. Experimental values for strain U2 are close to the theoretical. The greater departure of strain U1 from the theoretical can be explained as resulting from aggregation of the infectious units (Bald, 1950).

### *B. The Susceptible Site and the Initial Act of Infection*

The leaf must be injured before infection with virus is possible. This requirement can be illustrated by the fact that a leaf can be caused to take up relatively large amounts of liquid by the process of vacuum infiltration, so that the entire intercellular space of the interior of the leaf, ordinarily filled with air, will be occupied by liquid. Introducing tremendous concentrations of virus into the interior of a leaf by this means, and thereby placing virus in contact with nearly every cell of the leaf, has never produced a lesion in our experience and that of others (Caldwell, 1932). Yet, gentle rubbing of either surface of the leaf in the presence of virus will almost invariably produce lesions. Thus, the conclusion seems inescapable that an absolute requirement for infection is injury of the leaf, thereby producing a susceptible site through which the virus can enter the protoplasm of the host cells.

Returning to the infectivity-dilution curve, we see that the bending of the curve at high virus concentrations finds a reasonable interpretation in terms

of the susceptible site. The explanation is that, at high concentrations of virus, there is an insufficiency of susceptible sites to accommodate all the virus that may be present. With low concentrations, there is not enough virus to ensure infection of each susceptible site.

Kleczkowski (1950) has proposed that the leaf varies in regional susceptibility to infection. His hypothesis is derived from mathematical analysis of the infectivity-dilution curve, which indicates that the logarithm of the minimal effective concentration is normally distributed. One interpretation of this finding might be that some susceptible sites are infected with a single virus particle, whereas other susceptible sites require the cooperation of several particles to institute the infection. However, this interpretation seems to be eliminated by an exclusion phenomenon, which will be discussed later in this chapter. Another explanation might be that the susceptible sites differ in size so that there is a greater probability of virus making contact with a larger site.

The number of susceptible sites produced by rubbing can be appreciably increased by the use of abrasives incorporated with the inoculum (see Yarwood (1957) for references). However, even by this means, a maximum of around  $10^3$  susceptible sites per *N. glutinosa* leaf is about all that can be engendered, because more drastic and extensive injury results only in killing host cells before they reveal the process of virus reproduction.

For maximum lesion production, it is necessary for the virus to be present at the instant the susceptible site is produced. Rubbing the leaf first and then applying the inoculum later will result in fewer lesions than having virus present at the time of rubbing. Evidently, the susceptible site is in an optimum condition to accept the virus for only an instant and then rapidly loses the capacity. There are reports (Allington and Laird, 1954) that test plants deficient in potassium can still be infected with TMV for appreciable periods of time after rubbing the leaf. Yarwood (1957) has compiled the experimental results of comparison of different buffers, applicators, abrasives, host plants, viruses, etc. The reasons why one set of conditions will produce a greater, or lesser, number of lesions than another set are not very well understood.

### *C. Specific Infectivity of Tobacco Mosaic Virus*

Under most circumstances,  $10^{-9}$  grams of TMV per milliliter is about the minimum dilution of TMV that will start an infection either in the form of local lesions or as a systemic infection. Thus, the difficult question of why each infection requires in the neighborhood of  $10^6$  virus particles is posed; in this connection, TMV is more infective than any other plant virus.

Various interpretations have been advanced to account for the low specific infectivity of plant viruses. One of the arguments is that all virus particles

are equally infective but it is a rare event for a virus particle to be present at the exact location and the precise time when a susceptible site is produced. However, Rappaport and Siegel (1955) have obtained evidence to suggest that difficulty in creating susceptible sites is not the whole answer. Their findings indicate that a fraction of the particles identified as TMV in the extracellular state are simply not infective. The evidence comes from inactivation of the infectious activity of TMV by antisera. When the ratio of antiserum to virus is held constant, but the concentration of virus plus antiserum is varied over a ten-fold range, they find that the percentage of virus activity that survives the antibody treatment remains relatively constant. However, centrifugation experiments applying gravitational forces insufficient to sediment virus infectivity in the absence of antiserum revealed that the greater the total concentration of virus and antiserum, the more infectivity was sedimentable. Varying the total concentration of virus and antiserum led to the formation of different sized virus-antibody aggregates. They obtained evidence that the larger aggregates consisted of hundreds of virus particles, yet the fraction of infectious units in these mixtures was the same as in those mixtures containing the smaller aggregates. They believe that these results indicate that the number of infectious entities in a population of TMV is less than the total number of rod-shaped particles. Although some investigators do not concur with this interpretation of these particular results, a similar situation of low specific infectivity is known to exist in the cases of poliomyelitis and influenza viruses, due presumably, in part, to unavoidable thermal inactivation. It would be of interest to determine if similar results would be obtained with animal viruses as with TMV in controlled aggregation experiments.

#### *D. What Cells Serve as Entry Ports for Virus?*

As mentioned previously, the surface of an *N. glutinosa* leaf is composed of a heterogeneous collection of cell types. The question of what cells serve as the starting point of virus infection is still largely unresolved. Arguments have been presented which would appear to eliminate the leaf hairs as entry ports (Boyle and McKinney, 1938). Yet, as Sheffield (1936) showed more than twenty years ago, delicate puncturing of individual hair cells in the presence of virus would sometimes cause lesions to develop in the region of the injured hair cell. Unfortunately, the efficiency of starting infections by this means is very low. In our repetition of her experiments, we have seldom been able to get more than 5 % takes by this method, and I believe this to be the experience of others who have tried the same method. However, the conclusion that it is sometimes possible to start the infection in leaf hairs can scarcely be doubted. Whether or not the leaf hairs are a significant entry



point for starting the infection when the leaf is rubbed with virus is another question. I have found that it is possible to destroy most of the larger leaf hairs by rubbing the leaf in the absence of virus. When the leaf is rubbed 24 hrs. later with virus, the same number of lesions appeared as on control leaves which did not receive the rubbing pretreatment. Consequently, it is doubtful if the larger leaf hairs participate in starting the infective process when the normal technique of rubbing the leaf with virus is used.

The *N. glutinosa* leaf contains areas which are more difficult to infect than other areas. Utilizing a grid with 5 mm.  $\times$  5 mm. squares placed over a leaf, an analysis of the distribution of lesions has been made (Rappaport and Wildman, unpublished). Where the total number of lesions per leaf is in the range of about 10 to 100, there is an apparent correspondence between the number of observed lesions per 25  $\overline{\text{mm}}^2$  of area compared to the number predicted from a Poisson distribution, suggesting that lesions are distributed in a random manner. However, as the total number of lesions rises, the departure of the observed classes from the predicted increases enormously. For example, where approximately 1,000 lesions per leaf were produced by brush inoculation of the virus, the Poisson formula predicts the probability that an area will not contain a lesion as 1 in 10,000, whereas the observed frequency was closer to 1 in 100. Thus, we are forced to the conclusion that certain areas of the leaf are more resistant to infection than others.

As far as the very first act of infection is concerned, it is still impossible to say whether the susceptible site is produced only in a particular type of cell, or whether the potentiality is present in all cells, but realized only in a few because of the variability in ease with which susceptible sites can be produced.

#### *E. Nature of Virus Attachment at Susceptible Site*

A simple experimental observation indicates that when virus makes contact with a susceptible site, the union is essentially instantaneous and irreversible. In our experiments, we have never been able to reduce the number of lesions by vigorously washing the leaf immediately after inoculation by rubbing. Washing the leaf after rubbing with virus has long been a standard practice because of the report (Holmes, 1929) that the number of lesions is *increased* by washing. We have not been able to observe this effect. Rather, the same number of lesions appear, irrespective of whether the leaves are washed after rubbing or not. If the wash water is collected and tested for infectivity, lesions will be produced, but the amount of infectivity in the wash water is greatly reduced from that of the inoculum. The fact that infectious virus is obtained in the wash liquid suggests that such virus merely failed to make contact with the limited number of susceptible sites created

by the rubbing process. Those that did find the proper site were immediately "fixed" in such a way that they could not be removed by the washing process.

In this connection, Siegel (unpublished) has found that when infectious TMV nucleic acid is rubbed on *N. glutinosa* leaves and the leaf immediately dipped into a strong solution of RNAase, no reduction in lesions occurs. The enzyme was so concentrated as to cause almost instantaneous inactivation of the nucleic acid. This result serves as a further indication of the rapidity with which virus combines with the leaf and becomes protected from the vicissitudes of the extracellular environment. Further discussion of TMV infectious nucleic acid will be found in a later section of this chapter.

Inactivated virus does not compete with infective virus at the site of virus attachment (Rappaport and Siegel, 1955; Rappaport, 1957). Virus whose infectivity had been destroyed by ultraviolet irradiation did not reduce lesion counts even when as much as 10 parts of inactivated virus were present to 1 part of active virus at the time of rubbing. Since inactivation produces no observable change in the structure or composition of the virus, failure of inactivated virus to interfere with the attachment of active virus suggests that attachment is a subtle process that we are far from understanding. Some progress in this direction has come from a kinetic analysis of serological experiments that has led Rappaport (1957) to postulate that antibodies covering any one of about 10 critical positions on a virus particle render the particle noninfectious. Such coverage would constitute less than 1/100 of the total surface of a virus particle, a fact which further emphasizes the delicate nature of combination of virus and the susceptible site. Later in this chapter, evidence for an exclusion phenomenon will be presented which adds another complication to the understanding of the first process that sets virus activity in motion.

#### *F. Intracellular Development of Viruses as Deduced from Irradiation Experiments*

As the result of applying techniques developed by Luria and Latarjet (1947; Latarjet, 1953) for elucidating the sequence of steps in the process of infection by bacteriophage, it has been possible to gain some knowledge of the intracellular behavior of plant viruses immediately following the start of infection. The experiments are based on the notion that analysis of radiation inactivation curves can reveal changes in the state of the virus. The rationale behind the interpretation of radiation inactivation experiments is found in Stahl's treatment of the subject in Chapter 10 of this volume.

#### *G. Inactivation of Tobacco Mosaic Virus by Ultraviolet Light*

It has been shown (Siegel and Wildman, 1954) that different strains of TMV may vary about 6 times in their susceptibility to inactivation by ultraviolet

light when the virus is irradiated in a thin, liquid layer, and then rubbed on leaves to test its remaining infectivity. With strains U1 and U2, the fraction of virus particles surviving the radiation treatment is an exponential function of the dose of applied radiation. Such "single-hit" inactivation curves for the common strain of TMV had been found previously by Price and Gowen (1937), Oster and McLaren (1950), and Bawden and Kleczkowski (1953). Only the slopes of the straight line inactivation curves are different, as shown by the data plotted in Fig. 2. TMV strain U2 is much more sensitive to inactivation by radiation than is strain U1.<sup>1</sup>

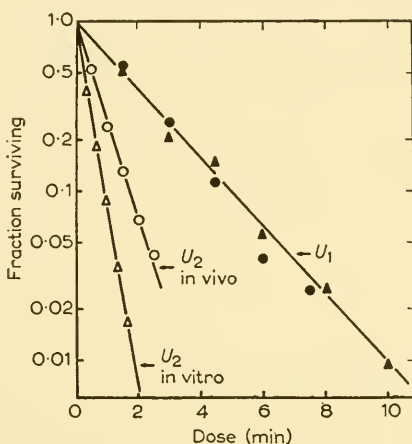


FIG. 2. A comparison of the survival curves obtained when TMV virus suspensions are irradiated *in vitro* with UV, and when leaves plus virus are irradiated soon after inoculation. Triangles represent fraction of virus surviving when irradiated *in vitro*. Circles represent number of lesions appearing on irradiated leaves plus virus. From Siegel and Wildman (1956).

Figure 2 also illustrates that nearly the same difference in sensitivity to inactivating radiation by strains U1 and U2 is maintained after virus has made contact with the host cells, for the same type of inactivation curves were obtained when the leaf was first rubbed with virus, and then the leaf and attached virus irradiated together. There is a small increase in slope for the U2 strain which remains unexplained, but the experimental points for the U1 strain can be fitted to the same inactivation curve, irrespective of

<sup>1</sup> An explanation for the difference in sensitivity between the U1 and U2 strains of TMV has been found by Siegel (1957) to reside in a difference in the bonding between the nucleic acid and the protein subunits of the virus particle.

whether the virus was irradiated before or after making contact with the host.<sup>1</sup>

Altering the concentration of virus does not change the exponential character of the inactivation curves. This indicates that an exclusion phenomenon operates to prevent more than one infectious virus unit from starting an infection at a susceptible site. Increasing the concentration of virus so as to present each susceptible site, on the average, with as many as 7 units of virus infectivity failed to change the exponential character of the inactivation curve, whereas it would have been predicted that if any of the 6 other units present at any given site could have substituted for the inactivated particle, the inactivation curve should have displayed a multitarget character. Apparently, only one unit of infectivity can institute the infection, and if, after making the proper contact with the susceptible site, its activity is destroyed by irradiation, other units at the site which may have escaped inactivation cannot replace the unit that was inactivated. We have no further information that would help to explain the nature of the phenomenon.

At first sight, the exclusion phenomenon would appear to conflict with the observations of Kunkel (1934), Lauffer and Price (1945), and Friedrich-Freksa *et al.* (1946), who find a small percentage of mixed lesions when two different strains of TMV are inoculated simultaneously on *N. glutinosa* leaves. We have also observed between 1 and 5 % mixed lesions when strains U1 and U2 are used as a mixed inoculum. However, I am far from convinced that mixed lesions *necessarily* arise from the entrance of both strains into the same cell. On the contrary, I have often observed that a single lesion can have its origin from two or more foci of infection. For example, when detached *N. glutinosa* leaves are inoculated with TMV and the leaves maintained under constant illumination at 20°C., tiny, dark specks (0.1 mm. or less in diameter), as viewed by transmitted light, will appear in about 18 hours after inoculation. Lesions develop from these specks. Occasionally, two such specks will be in such close proximity that the resulting lesion, when observed later, cannot be distinguished from lesions known to have their origin from a single speck. Indeed, with this consideration in mind, I would interpret the results of mixed inocula experiments as strong evidence in favor of an exclusion phenomenon. However, from Benda's experiments

<sup>1</sup> As far as TMV is concerned, photoreactivation by visible light of virus inactivated by irradiation has not been demonstrated, although Bawden and Kleczkowski (1953) have shown that tobacco necrosis virus is subject to photoreactivation. UV has a profound effect on the susceptibility of plant cells to virus infection. Irradiation of leaves prior to rubbing them with virus will greatly reduce the amount of infection. Exposure of UV-irradiated leaves to visible light results in photoreactivation of the susceptibility, for with a sufficient length of visible light treatment, infection will be as great as if the leaves had not been treated with UV (Bawden and Kleczkowski, 1952; Benda 1955; Siegel and Wildman, 1956).



(1956), it would appear that, under certain conditions, it is possible to cause a mixed lesion to arise from the infection of a single hair cell with two strains of TMV.

### *H. Changes in Virus Behavior during Early Stages of Infection*

When a delay is introduced between the time of rubbing the leaf with virus and irradiating the host-virus system, a significant change in both the slope and shape of the inactivation curve becomes apparent. Figure 3 shows

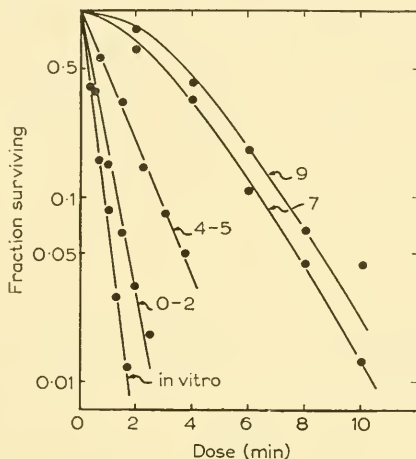


FIG. 3. UV survival curves of TMV strain U2 infective centers at different times after inoculation of a local lesion host, *N. glutinosa*. The points indicate the number of lesions on irradiated leaves plus virus compared with the number on unirradiated leaves. From Siegel and Wildman (1956).

the behavior of strain U2 in this respect. When irradiation is delayed 4-5 hours after inoculation, a marked change in the slope of the inactivation curve is observed, although the exponential character of the curve is still retained. Further extension of the time between inoculation and radiation to 7 and 9 hours produces a multitarget type of curve, indicative of the necessity of inactivating more than one infectious unit at each focus of infection before a lesion can be prevented from developing. Also noticeable is the fact that the final slopes of the multitarget curves do not return to the slope encountered immediately following inoculation.

Strain U1, being much more resistant to inactivation by radiation, has not shown as much detail in the changes in its behavior toward inactivating radiation as strain U2. The irradiation doses required to inactivate the U1 strain are too large to exclude the possibility that lesions fail to appear because of extensive damage to the host as well as the virus. However, we

have some information which indicates that a much longer period of time ensues after infection with U1 before a detectable rise in resistance to radiation is apparent. Whereas a change in slope of the U2 inactivation curve is apparent within 4 hours after inoculation, a similar change in resistance in U1 does not occur before 7–8 hours after infection. It has not been possible to decide on the time when the survival curve for U1 changes from an exponential to a multitarget character, except that it is later than for U2.

The results of these experiments have been tentatively interpreted in the following manner. After making contact with host protoplasm at a susceptible site, TMV passes through three different phases before multiplication of the virus occurs. During the first phase, which may last 2 hours at 20°C., or a shorter time at a higher temperature, the infectious entity shows no change in character from its extracellular behavior. The first phase of no change is then followed by a period of about 2 hours, in which the resistance to inactivation by radiation is markedly increased. The explanation we favor is that the virus changes its physical state.

Phase 2 is followed by another time period of about 1 hour in which no further rise in the resistance of the virus occurs. The meaning of phase 3 is not apparent, although it could be speculated that this is a period required for the infectious nucleic acid to find the proper structure within the host protoplasm required before self-replication can occur.

Phase 4 represents the start of the virus multiplicative process. While it would not be possible to claim precision, extrapolation of the final slopes of the inactivation curves to the ordinate during phase 4 suggests that the amount of infectious material doubles in amount in about two hours.

Of particular interest is the failure of the final inactivation slopes of phases 3 and 4 to return to the slope encountered in phase 1. The results suggest that the material that is multiplying is different in state than the virus particle which commenced the infection.

### *I. Inactivation of Tobacco Mosaic Virus Infectious Nucleic Acid*

The far-reaching discovery by Gierer and Schramm (1956) and Fraenkel-Conrat (1956), that the infectivity of TMV is a property of the nucleic acid portion of the virus particle has provided an additional means for experimenting with TMV during its earliest stages of reproduction in a local lesion host. First of all, it could be shown (Siegel *et al.*, 1956) that the differences in UV sensitivity between strains U1 and U2 in the extracellular state disappear completely when nucleic acid preparations are tested for viability after being irradiated, as shown by Fig. 4. Secondly, when the two kinds of nucleic acid are rubbed on a leaf, prior to irradiation of the host-virus system,

their subsequent inactivation behavior is the same, and closely similar to the sensitive behavior of Strain U2 (Siegel *et al.*, 1957).<sup>1</sup>

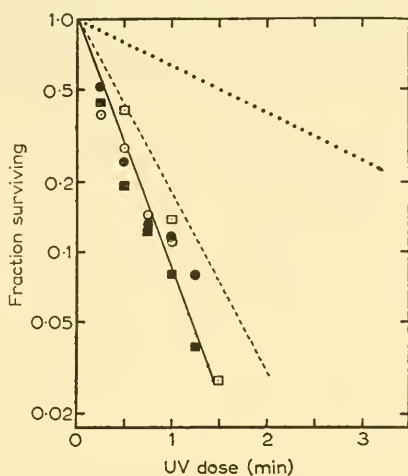


FIG. 4. Comparison of UV survival of infectious TMV nucleic acid irradiated *in vitro* and irradiating leaf plus nucleic acid immediately after inoculation. From Siegel *et al.* (1957).

Key: ○, U1 nucleic acid; ●, U1 nucleic acid infective centers; □, U2 nucleic acid; ■, U2 nucleic acid infective centers; —, U2; ---, U2 infective centers; . . . , U1 and U1 infective centers.

When a delay is introduced between the time of inoculation and time of irradiation, a remarkable difference in the behavior of the nucleic acids, as compared to intact virus, is noted, as shown in Fig. 5. Instead of undergoing the phases noted for strain U2, or the long lag period for U1 before resistance increases, the nucleic acids from both strains change their resistance to inactivation by ultraviolet light almost immediately after making contact with the host. Other findings (Siegel, unpublished) show that the exponential curve changes to a multitarget curve in a matter of only 3 hours after infection commences, in the case of the nucleic acids, as compared to the 7 hours or more for such a change to occur with the intact virus strains.

Thus, it seems clear that infectious nucleic acid can start reproducing more rapidly than intact virus, and we can feel some confidence in ascribing the first three phases in changing behavior of intact virus as associated with the freeing of the nucleic acid from the protein subunits as a preliminary step in the reduplication of the nucleic acid. Obviously, it would be of the greatest

<sup>1</sup> McLaren and Takahashi (1957) have also performed *in vitro* radiation inactivation experiments with TMV and TMV infectious nucleic acid. Where direct comparison is possible, there is a large measure of agreement on the experimental findings.

interest to know the answers to such questions as to whether some enzymatic action of the host uncovers the nucleic acid, or whether this can be accomplished by the virus itself, when placed in the proper environment. But further progress must await new ideas on how to locate the small proportion of cells that are sustaining virus activity out of the myriad that are as yet uninfected.

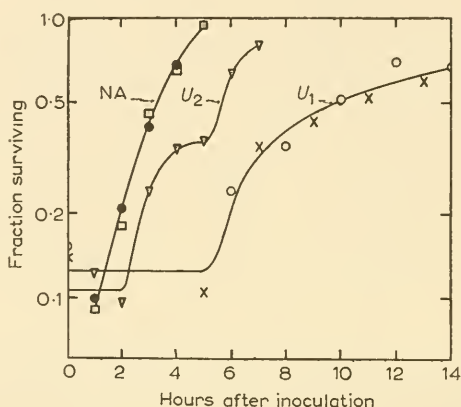


FIG. 5. UV survival of infective centers as a function of time after inoculation. Dose of UV: intact U2 and nucleic acids, 90 sec.; U1, 5 min. Note four phases in changing resistance of intact U2. From Siegel *et al.* (1957).

Key: ●, U1 nucleic acid; □, U2 nucleic acid; ▽, intact U2; ○ and ×, intact U1.

### *J. Spread of Infectivity from Focus of Infection*

The radiation inactivation experiments so far discussed provide information on the behaviour of TMV in presumably the first cell that is invaded by the virus, and which serves as the focus for infection of the many thousands of cells that comprise a visible lesion. A kinetic analysis of the spread of virus activity contained within lesions has presented further information on the behavior of TMV during its active period of reproduction (Rappaport and Wildman, 1957).

The essence of the experiments on lesion growth has been to maintain *N. glutinosa* plants, infected with TMV, under standard environmental conditions during the time that lesion growth measurements are being made. At 25°C., with 18 hours of light at 1500-foot candles during each 24-hour period, measurable lesions appear on a still expanding leaf about 40 hours after the leaf is rubbed with virus. The area of necrotic cells which distinguishes the lesion from surrounding healthy, green tissue continues to increase with time. While measurements reveal the lesions to be more in the shape of ellipses, rather than circles, the ratio of the major to the minor



semiaxes is not far from unity. The semiaxes have been averaged to produce the curves shown in Fig. 6 for three different strains of TMV. While there is a significant difference in the growth rate of the lesions produced by different strains, the rate at which the radius of a lesion increases is constant for each strain over the 5-day period that measurements could be made. Measurements beyond a 5-day infective period for the fastest growing strains are not practicable because of coalescence of lesions.

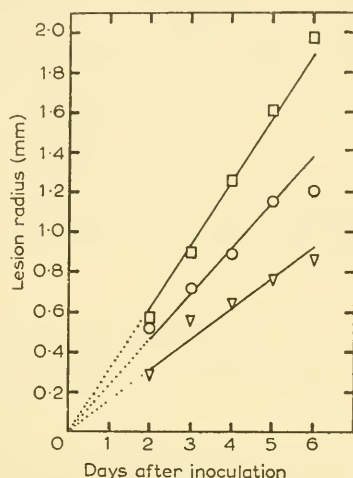


FIG. 6. Lesion growth of three different strains of TMV on *N. glutinosa* leaves. From Rappaport and Wildman (1957).

Key: squares, U1; circles, U2; triangles, Holmes' rib grass.

The conclusion is reached from these experiments that a perimeter of cells becomes necrotic and is added to the circumference of a lesion at a rate that can be estimated as involving 6 cells per day for U1, 4.5 for U2, and 3 per day for U8 (Holmes' rib grass).

There is a fairly sharp line of demarcation which sets the necrotic area apart from surrounding green tissue. However, microscopic examination of the green cells at the edge of the necrosis shows that obvious signs of degenerative changes are apparent in cells that are 3 to 4 cells in advance of the encroaching necrosis, in the case of strains U1 and U2. Strain U8 presents a different picture. The necrotic area is bounded by a layer of 5 to 6 cells which become greatly swollen in appearance. Often, the layers of swollen cells can be detected as a yellow halo surrounding the necrotic area of the lesion. Extending beyond the swollen cells, degenerative changes can be seen in a zone extending 4 to 5 cells further into the green tissue.

If the reasonable presumption is made that degeneration in the organization of cells as far removed as 4 cell layers from the necrotic area is a consequence of virus invasion, the entire time course of secondary cell invasion until the cell dies can be estimated. For U1, a necrotic cell is added to the lesion every 4 hours, and since virus activity extends 4 cells beyond the line of necrosis, it appears that 16 hours ensues from the time that a cell is invaded by U1 until the cell is dead and becomes part of the enlarging lesion. For strain U2, the time period from infection to death is 20 hours. A similar calculation leads to a figure of 80 hours for U8, but we are inclined to believe the estimate is unreliable because of the anomolous character of the edge of the U8 lesion, as compared to U1 and U2.

As shown in Fig. 6, extrapolation of the curves to the day of infection reveals no obvious deviation from a linear relationship during the time that lesions are forming, but are invisible to the eye, until the time that they can be measured. Consequently, we presume that the spread of the virus activity from the initial focus of infection is the same as that encountered during the visible enlargement of lesions.

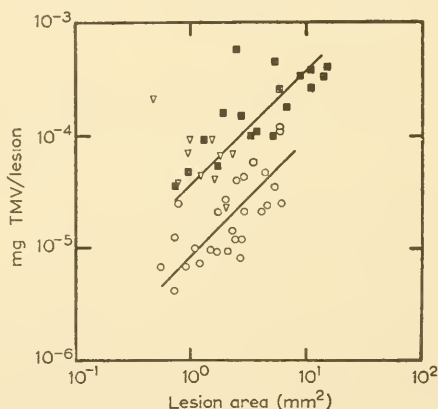


FIG. 7. Infectivity of three strains of TMV as a function of lesion size on *N. glutinosa*. From Rappaport and Wildman (1957).

Key: squares, U1; circles, U2; triangles, Holmes' rib grass.

### K. Yield of Virus per Infected Cell

As shown by Fig. 7, the amount of extractable infectivity is proportional to lesion area for strains U1 and U2. U8, in this respect, behaves differently, since the amount of extractable infectivity decreases as the visible lesion area increases. We have no ready explanation to account for the peculiar behavior of U8, except the reminder that the swollen cells at the

edge of U8 lesions may seriously complicate transfer of virus activity from cell to cell. Infectivity measurements have been utilized to estimate the yield of virus per cell. Typical results are shown in Fig. 8. While the points scatter

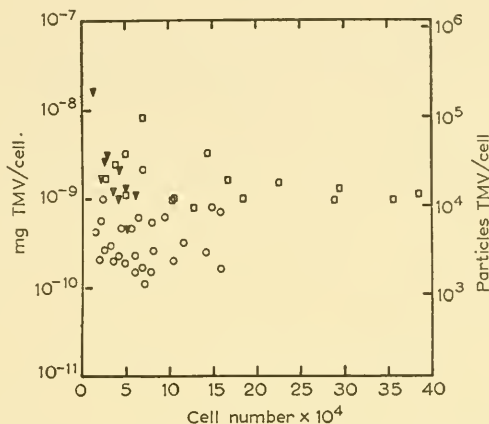


FIG. 8. Infectivity yield of three strains of TMV per cell, as a function of total number of necrotic cells. From Rappaport and Wildman (1957).

Key: squares, U1; circles, U2; triangles, Holmes' rib grass.

considerably, there seems to be a trend towards constancy of virus yield per cell during the entire course of lesion expansion for U1 and U2, although U8 appears to decrease in yield per cell as the necrosis enlarges. Evidently, we can estimate that in the 16-hour period that elapses from the time a cell becomes infected with strain U1 until it succumbs, between  $10^4$ – $10^5$  new virus particles are manufactured. For strain U2, the yield is about a magnitude less. A rough guess would indicate that an average doubling time of around 1 hour would have to be invoked to account for the  $10^3$ – $10^4$  virus particles per cell induced by U2 infection, a magnitude that is not out-of-line with the estimate derived from radiation inactivation experiments.

### *L. Nature of Infectious Material*

In what form is the infectious material transmitted from cell-to-cell? In the absence of any experimental evidence to the contrary, we are inclined toward the notion that infection is transmitted via nucleic acid. Because of the unique construction of plant cells, it seems clear that transmission by a lysis or leakage mechanism, as found for phage and animal viruses, is out of the question. Microscopic examination of necrotic cells indicates that the cellulose wall remains intact, but shrunken, after the cells die. Presumably, the cell wall still remains an impenetrable barrier to the passage of virus. We

are left then with the probability that TMV must travel via the protoplasmic bridges (plasmadesmata), which connect plant cells with each other, and which represent a route whereby the necessity for penetrating the cell wall could be circumvented.

Information on the size and frequency of plasmadesmata connections is too scanty and unsatisfactory to permit more than the impression that their diameter might be less than the long axis of an intact TMV particle ( $0.3\ \mu$ ), which would perhaps require orientation of virus rods before they could escape from one cell into another. I believe it is more plausible to think of transfer being accomplished by infectious nucleic acid, particularly since evidence from radiation inactivation experiments suggests that intact virus particles are not present during the earliest stages of the multiplication of the infectious material. One piece of unpublished experimental information may bear on the transfer problem. When the area of still green cells which surrounds the necrotic area of the lesion, but which is in the throes of degenerative changes, is dissected, and tested for the presence of extractable infectivity, the cells display much less infectivity than a comparable group of cells that are part of the necrotic area. Further confirmation of this finding could be interpreted to signify that nucleic acid has indeed invaded the cells and induced the degeneration as the result of its multiplicative activity, but the infectious material has not yet acquired a protective coat of protein subunits, with the result that the infectivity is vulnerable to inactivation during the extraction process. Consistent with this notion is the fact that infectious nucleic acid derived from extracellular TMV<sup>1</sup> is highly susceptible to inactivation by juices derived from ruptured plant cells.

*M. Is Tobacco Mosaic Virus Extracted from Lesions Identical with Tobacco Mosaic Virus Extracted from Systemic Hosts?*

Our knowledge of the chemical composition, physical behavior, and construction of TMV is, as far as I am aware, confined to virus that has been extracted from systemic infections. The question remains as to whether the virus formed in a lesion is in all respects identical with that derived from systemic hosts. We have some unpublished information to suggest conformity. By extracting a large number of lesions, the presence of rod-shaped particles could be ascertained by electron microscopy. The infectivity was removed from a solution by centrifugation in a manner comparable to the sedimentation behavior of TMV extracted from a systemic host. Lesion virus

<sup>1</sup> X-ray inactivation experiments by Ginoza and Norman (1957) and Lauffer *et al.* (1956), indicate the molecular weight of infectious nucleic acid to be of the magnitude of 2 million. Gierer (1957), on the basis of inactivation experiments with RNAase, also arrived at this order of size. Ginoza (1958) has presented a provocative conception of a TMV particle constructed from a single strand of nucleic acid.

is inactivated by a systemic TMV antiserum in a manner similar to systemic virus. The vital question of whether the specific infectivity, i.e., the number of particles per unit of infectivity, is the same or different for lesion versus systemic virus has not yielded a confident answer, although the results of one trial indicate no striking differences. We are inclined to believe in the identity of the two materials, but we would still like to see further experimental evidence to support this belief.

Thus, intensive study of TMV in its local lesion host has provided a reasonable working model for the intracellular behavior of a plant virus. The question now is how far these findings will apply to the systemic virus-host system, which is the subject of the next section.

#### IV. BEHAVIOR OF PLANT VIRUSES IN A SYSTEMIC HOST

##### *A. Quantities of Virus Synthesized*

Vastly more effort has been expended on understanding the behavior of TMV in systemic hosts than in local lesion hosts. The reasons are not far to seek. In a matter of a few days after a leaf has been rubbed with virus, symptoms will make their appearance on leaves above the leaf that was inoculated. Stanley (1937), shortly after his discovery that TMV could be isolated and crystallized, was able to show a millionfold increase in virus infectivity, as well as a concomitant increase in virus protein in the short span of 4 days after infection of tobacco leaves with TMV. In a matter of a few weeks, milligram quantities of virus can be readily extracted from each leaf showing symptoms (Cohen *et al.*, 1957). As far as the leaf that was inoculated is concerned, no very conspicuous symptoms will appear, although the leaf will also contain milligram quantities of virus some 14 days after inoculation. Thus, the ease with which newly formed virus can be measured is responsible for the emphasis that has been placed on the systemic system. In the experience of this laboratory, we have extracted as much as 70 mg. of virus per leaf within 20 days after infection, although an average value would be in the neighborhood of 5–10 mg. virus per leaf. Since the leaf weighed about 5 grams at the time of extraction, about 4 grams of which was water, the concentration of virus rose from about  $10^{-5}$  mg./ml. in the inoculum to around 10 mg./ml. in the leaf during 20 days of infection. On any scale of magnitude, TMV synthesis in the systemic host is in a class by itself. It is a remarkable facet of the TMV problem that a plant can endure such an enormous synthesis of a foreign, virus nucleoprotein without the plant showing distress. There is one strain of TMV which produces no symptoms whatsoever, and yet, extraction of the leaf two weeks after inoculation will yield around  $10^6$  particles of TMV per cell!



While it is true that many measurements of virus synthesis taking place in the systemic system have been made, most of such measurements throw very little light on the processes involved in virus multiplication at the cellular level of leaf organization. The problem of locating those cells in the systemic host that are directly engaged in virus activity is made even more difficult by the absence of necrotic lesions to serve as indicators of previous virus activity. Holmes (1931) has developed a staining method to reveal starch concentrations at the foci of infection, but this method has not been exploited to the extent of providing information on the intracellular development of virus. In time, slightly chlorotic areas which are considered to correspond to areas of virus activity will appear on the inoculated leaf. The most important progress toward elucidating virus behavior in the systemic host stems from the direct observations of infected leaf hair cells by Zech.

### *B. Direct Observation of Virus Activity in Hair Cells*

Zech (1952) has succeeded in infecting individual leaf hairs of the systemic host of TMV, *N. tabacum*, and then observing changes induced in these cells by virus. He has employed both phase and ultraviolet light microscopy. Much of his work is concerned with the systemic spread of virus infection from one organ to another, but these interesting observations will be omitted from this discussion.

Zech's observations, germane to this discussion, can be summarized as follows: (1) Large leaf hairs, consisting of 6 cells arranged in a row, the basal cell being an extension of the epidermis, are selected for infection and microscopic observation. The proximal cell is cut in the presence of virus contained in gelatin. (2) About 5 hours after infection, an accelerated rate of protoplasmic streaming is noticed in the first intact cell nearest to the point of infection. Within 10 hours, an increased rate of protoplasmic streaming is present in all of the hair cells. (3) About 60 hours after infection, a swelling of the protoplasm is detected, but the swelling occurs first in the basal cell, 5 cells removed from the point of infection. (4) About 74 hours after infection, disorganization of chloroplasts is observed in the basal cell, but disorganized chloroplasts do not appear in the cell next to the infection until around 100 hours have elapsed. (5) Amorphous masses, previously known in TMV literature as X-bodies,<sup>5</sup> appeared in the basal cell at 80 hours, but none

<sup>5</sup> As X-bodies and crystalline inclusions make their appearance, enormous numbers of rod-shaped particles can also be visualized by electron microscopy. Steere (1957) has isolated and sectioned crystals removed from leaf hairs and demonstrated a herringbone pattern of orientation of the rod-shaped particles. Nixon (1956) has sectioned leaf hairs and displayed astronomical numbers of virus particles *in situ*. Matsui (1956) has presented interesting electron microscopic observations of ultrathin-sectioned tissue infected with TMV.

appeared in the proximal cell during the entire experimental period, which extended to 216 hours. However, a crystal inclusion showed up in the proximal cell some 150 hours after infection. (It has been known, since Sheffield's experiments in 1939, that crystalline inclusions contain virus infectivity.) Zech was also able to follow similar changes in the cells radiating from the basal cell of the leaf hair and concluded that the changes were indicative of virus activity spreading slowly, in all directions, away from the basal cell.

The insensitivity of the TMV infectivity assay precludes the possibility for directly correlating infectivity measurements with microscopic observations of the leaf hair cells. Consequently, Zech was forced to resort to rubbing virus on the entire surface of leaves, and then, measuring for virus activity contained in leaf discs punched out at random from the infected leaves. In this way, he reached the conclusion that appearance of new infectivity was considerably delayed beyond the time when he could get microscopic evidence of virus activity in the hair cells. He postulates that the infective process begins by the virus entering an "eclipse" period of a minimum duration of 42 hours, and that, furthermore, the agent responsible for the transfer of virus activity from cell-to-cell is different in state from the TMV virus particles observed in the extracellular state.

Utilizing the ultraviolet light microscope, Zech (1954; Zech and Vogt-Köhne, 1955) have made additional fascinating observations of TMV activity in leaf hair cells. He finds that 4–20 hours after infection, the nucleus of the hair cell becomes surrounded by UV-absorbing material. This first stage is followed by increased UV absorption in the cytoplasm, accompanied by a diffusion of the material that was concentrated around the nucleus. This stage is accompanied by a reduction both in the volume of the nucleus and its contained UV-absorbing material. With the appearance of X-bodies between 42–220 hours following infection, which is also the period before infectivity can be ascertained by the method described above, the nucleus begins to regain some of its lost volume. When the infection period progresses to the point where crystals arise out of collapsed X-bodies, the nucleus and nucleolus may swell up to four times their original size.

Zech's results, at first sight, would seem to indicate a distinct difference in the process of infection of TMV in the systemic host compared to the local lesion host, particularly in terms of the 42-hour minimum eclipse period for the systemic infection. In the local lesion system, multiplication occurs 7–9 hours after infection, and only about 16 hours elapse from the time the virus invades a new cell until the cell becomes necrotic, after about  $10^4$  new particles of virus activity have been manufactured. Analysis of the results of Goodchild and Cohen, and Yarwood may help to throw some light on this seeming great difference.

### *C. Course of Virus Synthesis in Directly Inoculated Leaves*

Goodchild *et al.* (1958) made simultaneous measurements of the relative amount of infectivity, the number of particles detectable by electron microscopy, and the amount of specific virus nucleoprotein that could be extracted from directly inoculated leaves of *N. tabacum* var. Turkish Samsun as a function of time, after rubbing the surface of the leaves with a solution of TMV. The results of the infectivity determinations are presented in Fig. 9.

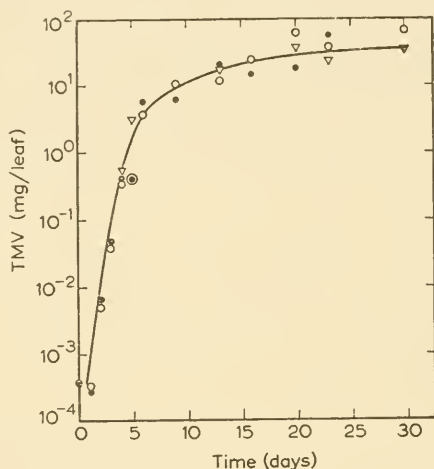


FIG. 9. The relative infectivity that can be extracted from directly inoculated leaves of *N. tabacum* var. Turkish Samsun as a function of time of infection with TMV strain U1. Different kinds of points represent repetitions of bioassay for infectivity. From Goodchild *et al.* (1958).

(The three methods showed a close correspondence throughout the period of infection, leading to the conclusion that the virus does not change in specific infectivity.) The inoculum contained  $10^{-2}$  mg. of strain U1 TMV/ml. An increase in relative infectivity could be detected in these greenhouse-grown plants two days after rubbing. As time of infection continued, a steady rise in relative infectivity ensued until a marked flattening of the curve set in about 8–10 days after the start of infection. Steere (1951) has found virus increment curves of similar shape for both TMV and bushy stunt virus by counting newly synthesized virus particles by means of electron microscopy. The increment curves flatten in lesser time than that shown in Fig. 9.

The great probability that only a minute percentage of the total number of cells comprising the surface of a leaf becomes infected at the instant of inoculation almost automatically leads to the conclusion that a curve such as Fig. 9 is a composite of at least two processes, (1) the multiplication of virus within individual cells, and (2) the spread of virus into other cells, together with multiplication in these cells.



Presuming the data plotted in Fig. 9 to be preponderantly a reflection of the rate of spread of virus from cell-to-cell, an estimate of the spreading rate on a cellular basis can be made and compared to the rate ascertained by measuring lesions in the local lesion host. The leaves that were rubbed had an area of about 150 cm.<sup>2</sup> The concentration of virus,  $10^{-2}$  mg. TMV/ml., would lead to the expectation that the infection probably arose from about  $10^3$  foci of infection. The surface of the leaf is estimated to contain  $10^8$  cells. If the bend in the logarithmic curve at 10 days represents the time when most of the cells on the surface have been infected, we arrive at the notion that  $10^5$  cells become infected from each focus in 240 hours. The area occupied by  $10^5$  cells would be about 15 mm.<sup>2</sup>, or a radius of a circle of 2.3 mm. If the average cell diameter is about 30 microns, and the radius increases at the constant rate of 230 microns per day, we arrive at the figure of 8 cells per day that become infected along a radius extending from a focus of infection. Crude as the estimate may be, it suggests that the rate of spread of virus infectivity in the two different hosts does not differ by a large amount.

In 1934, Uppal measured the time required for TMV infectivity to travel through the cells between the upper and lower epidermis of *N. sylvestris* leaves, the latter being another systemic host for the common strain of TMV. The rate of movement in this direction was found to be 7 to 8 microns per hour, which is the same order of magnitude estimated from the experiments of Goodchild *et al.*

Yarwood (1952) has determined the earliest time that an increase in amount of infectivity can be detected after rubbing *N. tabacum* leaves with the common strain of TMV and tobacco necrosis virus. While there is considerable scatter of the experimental points, when all of the results of a large number of trials are plotted as the logarithm of amount of infectivity versus time after inoculation, the pattern is clear. For about 8 hours after inoculation, no change in virus infectivity occurs. Then, from the 8th to the 14th hour, an exponential rise in the amount of infectivity occurs. Thus, Yarwood proposes that TMV undergoes a latent period of about 8 hours, and then the infectivity begins to multiply such that the amount doubles with each succeeding hour. Tobacco necrosis virus undergoes a longer latent period and doubling time. Since Yarwood's common strain of TMV is probably identical with strain UI, it is significant to note that the latent period in the systemic host corresponds very nicely with the time before an increase in resistance to inactivation by radiation can be detected in the local lesion host.

Thus, we have two pieces of evidence, the latent period \* and the rate of spread of the infectivity from cell-to-cell, which imply that the fundamental

\* Note added in proof: Schramm and Engler, *Nature*, 181, 916 (1958), have found a latent period of 30 hours for TMV in *N. tabacum*. The latent period is shortened by 10–12 hours when the infection is commenced with TMV infectious nucleic acid.

behavior of TMV as an infectious entity is similar in both the local lesion and systemic host. Considering this evidence, I would therefore suggest that Zech's observations on virus activity in leaf hairs depict an interesting special case of virus activity in a particular type of leaf cell, but the question remains as to whether they are applicable to virus behavior in other cells of the leaf.

In connection with virus activity in leaf hairs of *N. glutinosa*, Dr. Morris Cohen and I have made repeated attempts to cause lesions to arise as the result of wounding individual leaf hairs in the presence of TMV. Similar to the findings of Sheffield (1936) and Hildebrand (1943), an occasional lesion would appear, but the frequency of success was miniscule. When more precise wounding was attempted with a micromanipulator, success dropped to zero in more than 100 trials. We have also tried to infect detached hair cells with no success, even though the hairs would continue to show evidence of protoplasmic streaming three weeks after detachment. In other experiments, we have plasmolyzed the hair cells before detachment, so that a cut could be made through the cell without disturbing the protoplast, but leaving the protoplast directly exposed to the virus-containing medium. The vitality of the cell is unharmed, as judged by continuance of protoplasmic streaming, but we found no evidence to suggest that exposure of the naked protoplast to TMV or to infectious nucleic acid, led to earlier disorganization of the protoplast than controls in the absence of virus. Exposed protoplasts remained streaming for about four days, or twice the time required for the development of a respectable lesion. Consequently, I wonder if hair cells on *N. glutinosa* leaves even participate in virus infection, except perhaps to transport the infectious material to other cells where the infectivity can multiply.

#### *D. Necrosis and Phloem Cells*

How can we account for the fact that the vitality of a cell invaded by virus in the local lesion host is destroyed, whereas in the systemic host the cell remains alive? Further, how can we account for so much less virus being synthesized in the cell that dies compared to the cell that lives? I am tantalized by another of Zech's observations (1952) that might provide a clue leading to an explanation. He observes signs of virus activity in every kind of cell in the leaf except phloem cells. Phloem cells form a part of the tissue responsible for transporting organic materials, such as sugars formed by photosynthesis, from the leaves to other parts of the plant, and supply organic materials necessary for the integrity of leaf cells. They are living cells terminating in the vein endings of leaves which abut on mesophyll cells. There is much evidence to show that virus is spread in the systemic host via the phloem (Bennett, 1940), and that virus transport is a passive affair. Virus can pass out of the leaf through long distances of stem to enter other leaves, without

necessarily invading the cells that enclose the phloem.<sup>1</sup> I suspect that virus does not impair the normal functioning of phloem in the systemic host, but kills the phloem in the local lesion host. Impairment of the phloem may occur before the infectivity can be transported, with the result that the virus is, perforce, confined to the lesion. Preliminary microscopic observations, made by Warren Furumoto in my laboratory, indicate that disorganizational changes in phloem cells can be observed in the proximity of an encroaching lesion. Such changes suggest that loss in phloem vitality precedes necrosis of the mesophyll cells in contact with the phloem element. It could be imagined, also, that mesophyll cells in the presence of impaired phloem are somewhat analogous to an animal with impaired kidney and liver function. The cells can no longer obtain or discharge the products normally transported by the phloem. Thus, the possibility exists that necrosis of the cell is not the direct result of virus multiplication, but is secondary in origin, and that the local lesion host cell dies before reaching its full potential for virus synthesis. It is also significant that with some other plant viruses, such as curly top, wheat streak, etc., Esau (1956) finds phloem necrosis to be the first evidence of the presence of virus.

Holmes' recent review (1954) provides many examples of genes controlling the type of host response produced by plant viruses. In tobacco, a single, dominant gene is sufficient to determine whether TMV in an *N. tabacum* plant will be confined to local necrotic lesions or will become systemic. Extending the above speculation, it could be imagined further that gene action, in this instance, determines whether or not the virus can multiply in the phloem.

#### *E. Effect of Virus Synthesis on Composition of Host Proteins*

The changes in host protein composition induced by TMV in a systemic host have been the subject of several investigations (Wildman *et al.*, 1949; Commoner *et al.*, 1952; Bawden and Kleczkowski, 1957); it is to be regretted that there is still nonuniformity of results of the various investigators. We have repeatedly found that, as the virus in directly inoculated leaves increases in quantity, there is a corresponding decrease in soluble host proteins. Other workers, applying the same technique of electrophoresis for measuring virus and host proteins, arrive at the conclusion that no such relationship can be clearly demonstrated. The discrepancy may be due to the use of different kinds and concentrations of buffers during the extraction of the leaf proteins.

<sup>1</sup> Cohen *et al.* (1957), on the basis of experiments dealing with competition between strains U1 and U2 in a systemic host of TMV, have presented a theory to account for the origin of the systemic infection in leaves other than the leaves that were inoculated.

### *F. X-Protein*

An important new development in the study of TMV in its systemic host has been the discovery by Takahashi and Ishii (1952) and confirmed by several other workers (Jeener and Lemoine, 1953; Commoner *et al.*, 1953) that synthesis of TMV nucleoprotein is accompanied by the appearance of other proteins which are closely related in amino acid composition and serological properties to TMV. The X-protein of Takahashi and Ishii does not contain phosphorus and is devoid of nucleic acid, although Bawden and Pirie (1956) claim that similar materials prepared by them do contain small amounts of P. The protein can be aggregated into structures which appear under the electron microscope to be identical in shape with the TMV particle. Discovery of X-protein has provided a decisive clue to the construction of the TMV particle (Franklin 1955).

Two possible origins of the X-protein have been considered. One notion, that the X-protein arises through the disruption of originally intact TMV particles due to the harshness of the extraction process, seems to be eliminated by the finding that subjecting purified TMV to the extraction process does not produce X-protein (Takahashi and Ishii, 1953). The other idea, that X-protein represents a stage in the synthesis of TMV finds support from isotope experiments. Delwiche *et al.* (1955), using isotopic nitrogen, indicate that late in the period of infection the isotope is incorporated into X-protein at a faster rate than into TMV, indicating that synthesis of X-protein precedes synthesis of TMV. However, it is my opinion that the broad conception now gained of the reproductive behavior of TMV and infectious TMV nucleic acid would logically place the formation of protein subunits after reproduction of the nucleic acid. If X-protein is identical with the subunits, we might imagine a lag in their production at first, and then, perhaps, an acceleration to the point where they are in surplus late in the total infective period. We are left with the vital question, however, of how the nucleic acid manages to engender its own formation as well as that of the protein, about which plant virology is still in the dark.

## V. SUMMARY OF STEPS INVOLVED IN PLANT VIRUS REPRODUCTION

Taking the experimental evidence as a whole, the sequence of events leading to the multiplication of a plant virus, as represented by TMV, can be summarized in the following way:

1. TMV gains access to host cell protoplasm only as a result of a wound that exposes the host protoplast and permits the infectious material to surmount the otherwise impenetrable barrier of the cell wall. Producing the right kind of wound is a delicate and subtle process whose precise nature is



not known. Of the  $10^7$  to  $10^8$  cells on the surface of a leaf, all of which seem to be able to support virus activity, only about  $10^3$  of them may be infected at one time under the usual conditions of virus inoculation.

2. Attachment of extracellular virus to the host protoplast is an irreversible and practically instantaneous process. An exclusion phenomenon operates at the site of attachment such that only a single infectious entity is responsible for the processes which will ultimately lead to the formation of a lesion.

3. After attachment to the host cell protoplasm, the virus undergoes sequential changes in its sensitivity to inactivation by radiation, which are considered to involve the release of nucleic acid from the virus particle. Multiplication of the nucleic acid then ensues.

4. Once multiplication is underway, newly formed, infectious units invade adjacent cells by way of the protoplasmic strands which interconnect all of the cells of the leaf. The spread of infectious units is continuous. After the virus has left the cell which served as the focus of infection, about 16 hours elapse between the time of infection and the necrosis of another cell in a local lesion host. During this interval, around  $10^4$  rod-shaped particles are synthesized in the infected cell. Of the large number of particles manufactured, only a fraction of these possess the information necessary to initiate a new round of virus reduplication as the cycle of extracellular  $\rightarrow$  intracellular  $\rightarrow$  extracellular virus is repeated in a new host plant.

#### ACKNOWLEDGEMENTS

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## Chapter II

### The Biochemistry of Plant Viruses

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## I. INTRODUCTION

The biochemical study of plant viruses is still very largely in its early stages. For many years the plant viruses were regarded as unusual, somewhat ethereal, entities having properties which were more characteristic of the supernatural than of the chemical laboratory. Nowadays, largely owing to the pioneer work of Stanley and of Bawden and Pirie, the plant viruses are commonplace chemicals used for calibrating physical apparatus of various kinds and used as sources of nucleic acids. They are now being investigated by all manner of methods from X-ray crystallography and electron microscopy to enzymatic and immunological tests, and yet we still do not know why viruses cause disease. Generally it is thought that the viruses cause the diseases in plants by sequestering the synthetic potentialities of the plant. Nothing could be further from the truth. The damage caused by virus infection to plants may range from negligible, or only detectable by careful statistical analysis, to complete death of the plant, while the amount of virus in a plant may range from grams per kilogram to minute amounts. Often, in fact usually, severity of disease is inversely correlated with the quantity of virus produced. It is quite evident that the severity of the disease has little or no connection with the amount of virus which is being synthesized or which is present at any one time. As the viruses evidently produce their effects by some kind of chemical mechanism, it is obvious that a mere study of the constitution of viruses will not give us more than a very superficial idea, if any, of the way in which viruses produce their pathological effects.

The study of plant viruses has followed a more or less logical development. They were first characterized as nucleoproteins at a time when it had become accepted that the enzymes were protein in nature, and the general interest in the viruses was largely centered around morphology. Much labor was expended, and still is being expended, in determining the morphology of viruses by indirect methods, such as viscometry, light scattering, flow birefringence, diffusion, and so on. Some of this produced useful information, but much of it is now purely of historical interest, because much of the morphological work is better done by electron microscopy.

The protein components of viruses attracted much attention for many years, and attempts were made to cause mutations and otherwise to interfere with virus activity by modifying the proteins. Nowadays it is believed that the nucleic acids are the prime factors and much the same is being done with them. The protein parts of viruses are not being neglected, however, and an all-out assault is at present being made on the chemical composition of the simpler viruses. Far from being a routine task, this is proving interesting and indeed surprising. A short time ago it was thought that viruses contained only protein and nucleic acid. Now odd structures are being recognized at

frequent intervals, and it seems certain that many of our ideas about the constitution even of the best understood viruses will have to be revised.

The present concept of the smaller plant viruses is that they consist of tubular or globular bags made up from small protein subunits of a limited variety, and enclosing a central mass of ribonucleic acid which may consist of one chain, or a limited number of chains, of nucleotides. More and more the protein subunits, either singly or as polymerized protein resembling the virus itself, are being encountered in infected plants. This phenomenon is clearing up some of the anomalous results which have been reported in the literature, and study of these proteins may lead to better understanding of virus multiplication.

The following chapter is an attempt to summarize some of the present information about those plant viruses which have been studied sufficiently to give a consistent picture. An attempt has also been made to point out some of the pieces of information which are missing. In this, many of the plant viruses which have been studied have been omitted from discussion because the information that has been obtained about them is trivial or incomplete. Many plant viruses, too, are only known from the disease which they cause, and possibly also from electron micrographs. A number of viruses which have been purified and even crystallized are also omitted because none of the work on them has yet been published.

## II. THE PURIFICATION OF PLANT VIRUSES

The problems of isolating plant viruses from diseased plants are numerous and far from completely solved. Plant cells in general consist mainly of a large vacuole filled with cell sap and surrounded by a thin layer of protoplasm in which are embedded the cell nucleus and the various plastids. It is not known with certainty how the virus is distributed in such cells, although it is probable that it develops in the cytoplasmic layer, and it is certain that, in the case of the tobacco mosaic virus, a large proportion of the virus particles is eventually deposited inside the vacuolar sap in the form of crystalline masses (Fig. 1). The problem then is to remove the virus from as much of the plant material as possible and with the minimum loss. This has only proved practicable because many viruses have such unique physical and chemical behavior that it has been possible to destroy or otherwise remove extraneous matter from them by relatively simple procedures. However, lest it be thought that the isolation procedures for obtaining virus particles from plants have been perfected, it is as well to realize that less than 10 % of the known plant viruses have been purified to date.

The first requirement for the purification of a plant virus is that there should be a source of material containing sufficient virus to make the isolation

practicable. At present the limit is of the order of 5 to 10 mg. of dry virus per kilogram of fresh leaf material, but for consistent successful purification a larger quantity than this is desirable. This very factor limits the viruses susceptible to biochemical study to those few which are exceptionally infectious, and which indeed may not be wholly representative of plant viruses as a whole. In addition to this difficulty of isolation, which may well be overcome by the use of novel techniques, present-day methods of physical and chemical investigation, highly developed as they are, require at least 2 milligrams of pure virus material, so that the problem of purifying many of the interesting plant viruses also becomes one of dealing with relatively vast volumes of material.

### *A. Growing the Virus-Infected Plants*

In addition to the requirement of a source containing a substantial quantity of the virus material, there is also a requirement for certain properties in the plant sap as expressed from the virus-infected hosts. Because of this, plants which contain large amounts of gums, latex, or which have unusually large quantities of phenolic constituents are to be avoided. In fact even such conditions as the age and maturity of the host plants are exceptionally important. Thus, for example, in the purification of virus from tobacco plants, young, vigorously growing plants with a growth which horticulturalists would term "soft" are to be preferred, although this is not always true. Young plants also tend to contain less pigment, and, in general, are easier to deal with than are older plants. For routine purification work, plants are best raised from seed, when they are usually, though not invariably, free from virus disease, and transplanted singly at the cotyledon stage, or shortly afterward, into individual 3-inch diameter pots containing a sterilized compost which has an adequacy of nitrogen, preferably in the form of an insoluble organic substance like ground horn, phosphate, and potassium, as well as vermiculite or peat to ensure a sufficiency of water. The plants are best grown under glass and inoculated when small: thus, tobacco plants should be some 6 inches from leaf tip to leaf tip when inoculated with a virus giving a systemic infection. For viruses such as the tobacco necrosis viruses which cause only local infections, the leaves to be inoculated should be about 5 to 6 inches long and sufficiently mature to have a hard, somewhat smooth surface, because the younger, soft leaves are not so susceptible. The leaves are harvested after maximum virus content has been reached, which will vary with the virus, the plant species, climatic conditions, etc., and which must be determined empirically.

The harvested leaves may then be sorted into those most likely to contain large amounts of virus. This procedure, coupled with the removal of leaf



ribs, may increase the yield from a given weight of starting material several-fold, and is of advantage when the yields expected are small. In general, however, this is a waste of time, and when working with viruses, such as the tobacco mosaic viruses or turnip yellow mosaic viruses, it often pays to throw away a large proportion of the virus in the plants and to gain in the ease of handling of the larger volumes of material involved.

### *B. The Handling of the Virus-Containing Sap*

In order to extract the virus from the "sap" of the plants, which amounts to some 80 % of the weight of the fresh leaves, the leaves must be subjected to gross damage. This is best accomplished by means of a domestic meat grinder, preferably electrically operated, and with a worm which compresses the material before it reaches the cutters. Such grinders are obtainable commercially, but with difficulty. The use of the more recent blenders is not recommended because they are not at all suited to dealing with plant materials.

When the plant cells are ruptured the virus becomes suspended in a medium which is generally referred to as "sap." It is important to recognize that this material is essentially a pathological fluid, in which the virus is subjected to a change in its osmotic environment, and which contains a miscellany of enzymes and their substrates, and intracellular materials in various stages of disintegration. Many viruses tolerate such an environment reasonably well, and it is not usual to add materials to stabilize the osmotic pressure of the sap, although this might be profitable in case of the larger plant viruses. Much of the solids in the leaves may be eliminated before the actual crushing of the cells by freezing the leaves to about  $-10^{\circ}\text{C}$ . for a day or two before they are actually ground, and this may also assist in the grinding, because many leaves are more easily disintegrated in the frozen state. The leaf tissue is then allowed to thaw and the sap expressed through muslin or some other coarse cloth. On a large scale the sap may be obtained by means of a basket centrifuge or a large screw press.

When the plant sap is exposed to air, the phenolic substances which it contains tend to become oxidized to quinones, which combine with proteins including the virus. This may be prevented by the addition of a reducing agent, such as  $\text{NaHSO}_3$ , during the grinding, some 0.1 % being sufficient, or by adding a small quantity of ethylenediamine tetraacetate (Versene), or cyanide, which inhibits the metal catalysis involved in these oxidations. Bisulfite is preferable, because some procedures of purification take advantage of the presence in the sap of divalent cations such as  $\text{Ca}^{2+}$ , which would be removed by the ethylenediamine tetraacetate. Usually, if sufficiently young, actively growing plants are used, trouble from quinone formation is not serious, and the addition of substances to prevent it is not necessary.





FIG. 1. Composite photomicrograph of a hair cell of tobacco showing four crystalline inclusions of tobacco mosaic virus. Phase contrast photomicrograph (Magnification:  $\times 500$ ).



FIG. 2. Picture of a centrifuge tube in which turnip yellow mosaic virus was centrifuged for 2 hours at 12,000 r.p.m., showing separation into boundaries. Photograph by scattered light.



### *C. Clarification*

The most important single stage in virus purification is the clarification of the sap. Even centrifuging is not adequate for the removal of the pigments, proteins, and other large particles which it contains; therefore it is necessary to subject the sap to some chemical or physical treatment to eliminate most of these substances. Furthermore, it is necessary to do this at this early stage in the purification, because plant proteins become less easy to get rid of as the isolation proceeds.

There are only two methods which are of general use in clarification. One is particularly satisfactory, although it has not been employed until recent years: that is the addition of 300 ml. of 90 % ethanol to each liter of strained sap with vigorous stirring. This procedure causes the immediate formation of a coagulum which may be centrifuged off at low speed, leaving a golden, slightly cloudy fluid which contains the virus. A skin of chlorophyll-containing fatty material may form on the top of the fluid when it is centrifuged, but this may be removed by pouring the supernatant fluid through muslin. There are two contraindications to the use of ethanol for clarification. There may be viruses which cannot tolerate this level of ethanol, which is as low as will permit successful clarification, but so far none are known to be so very sensitive. The other is that the virus may be precipitated by weak ethanol at the pH of the sap (for most plants this is somewhat on the acid side of neutrality). This possibility has to be considered because, at a slightly higher ethanol concentration, the turnip yellow mosaic virus is precipitated at between pH 4 and 5, and this was indeed part of the original purification procedure for this virus.

The other useful method for clarifying sap depends upon the fact that when the latter is heated to 55°C. a coagulum forms almost instantly and may then be removed by centrifuging. Many viruses will tolerate 55°C. for a short time, and so this is a rapid and convenient procedure. Some viruses are intolerant of these temperatures, and some saps, such as those from cruciferous plants, do not clarify readily by heat. It is also significant that plant pathologists have found that the "thermal inactivation point" i.e., the temperature which a virus in expressed sap will just not tolerate for 5 minutes (a concept which is not altogether scientific), of many viruses which occur in low concentration is of the order of 55°C. It is now known that in at least one of these instances the virus is not so unstable when purified, and it is probable that the "inactivation" is actually due to the entrainment of the virus in the coagulum. This possibility of entrainment is ever present and probably accounts for much of the difficulty in purifying many viruses which are present in low concentrations.

Another popular clarification procedure, and one which would be better abandoned, consists of the addition of a basic substance, usually  $K_2HPO_4$ ,

in an amount sufficient to make the final concentration some 1 % (w./v.). This causes a coagulum, which is to some extent dependent upon the presence of divalent cations in the sap and the virus is left in the supernatant fluid. In some cases quite severe losses of infective material ensue and the method has little to recommend it apart from respectable antiquity.

The most recent innovation in the preparation of clarified sap is the use of butanol in the purification of the tobacco ringspot virus. This procedure, which resembles closely that adopted by Morton (1953) to release soluble enzymes from lipoprotein complexes in mammalian tissues, will be discussed elsewhere, but it is of interest to note here that it is evidently not devoid of risk, because it was found to eliminate the protein associated with the turnip yellow mosaic virus nucleoprotein, which is not a normal plant constituent and which has many of the properties of the virus itself.

There are, of course, innumerable other ways in which sap clarification might be achieved. Thus, for example, with those viruses which are not precipitated by 0.4 saturation with  $(\text{NH}_4)_2\text{SO}_4$ , this salt may be used for the clarification of the sap.

#### *D. Centrifuge Separation Techniques*

The use of centrifuges for virus purification is usually fairly straightforward and routine. Provided that the centrifuge is running smoothly and at constant temperature, boundary formation takes place and the bulk of the virus is sedimented in a finite time, which can be calculated accurately for a sectorial cell, and approximately for other types of cell. Sometimes, however, it is necessary to perform separations which are quite difficult and which require special techniques. As an example, both the turnip yellow mosaic virus and the wild cucumber mosaic virus preparations contain two constituents, which had to be separated for identification, and the only obvious method for effecting this separation was by means of centrifugation.

##### *1. Turnip Yellow Mosaic Virus*

In the case of the turnip yellow mosaic virus two components are present having sedimentation coefficients approximately in the ratio of 1 : 2. Consequently, if one were to sediment the faster component completely, only about half the slower component would be in the pellet. A second sedimentation would leave one-quarter, and so on. Thus in theory one cannot obtain the faster sedimenting nucleoprotein component completely free from the slower protein component by simple sedimentation. Yet in practice this separation is quite simple. The reason for this is as follows: In an ordinary centrifuge, the boundaries are in a metastable condition. It is not generally appreciated that, because the centrifugal field increases with the distance

from the axis of rotation, the regions between the boundaries in a cell or tube are in a constant state of mixing. This is, of course, the reaction in the fluid to the centrifugation, which tends to cause a decrease in concentration with increasing radius which has to be eliminated by convection. In addition to this inevitable stirring, there is a tendency for the periphery of centrifuges to heat more by air friction. This causes a decrease in density of the fluid at the bottom of the tubes, and so also gives rise to stirring. Because of these effects, and because of vibrations due to imperfect balancing, the boundaries tend to be unstable. In a good ultracentrifuge stability is achieved down to very low concentrations, but in ordinary angle centrifuges running in air, boundaries cannot remain stable if the concentration is less than a few milligrams per milliliter for substances having sedimentation coefficients of less than 100 S. When this happens the rate of sedimentation drops off rapidly.

When this takes place most of the slower component remains in the supernatant fluid suspended by the convection in the centrifuge tube in much the same way as a piece of thistledown is suspended in a breeze. The more rapidly sedimenting component is not so much affected by the stirring especially if it is present in greater amount at the start. The pellet which forms is almost pure, but the supernatant fluid is, of course, a mixture.

The more slowly sedimenting component may be isolated in a reasonably pure condition by spinning the mixture in sufficient concentration for two boundaries to form (Fig. 2). The upper layer of material may then be sampled by means of a pipette with a fine tip. This method is ideal for making material of 80 to 90 % purity, but 100 % purity cannot be realized; the only way to attain this is by an ultracentrifuge cell having a filter paper layer at the bottom (Markham, 1953b) or by means of the method of gradient centrifugation.

## 2. *Gradient Centrifugation*

A technique which has great potentialities in the separation of unstable viruses and for the separation of mixtures, such as the two components of turnip yellow mosaic or wild cucumber mosaic viruses (q.v.), is the density gradient centrifugation method developed by Brakke (1953, 1956).

The idea of using artificial density gradients to stabilize centrifugation and electrophoresis is one which has occupied the minds of workers for a long time, but the full potentialities of this method were not realized until the S.W. bucket rotors were developed by Spinco. Brakke took advantage of these rotors for his experiments, and he proceeded roughly as follows: In each tube equal volumes of 40, 35, 30, down to 5 % sucrose or an equivalent series in buffer solution are layered successively. One then obtains a variation in density from 1.02 at the top of the tube to 1.17 at the bottom. The density



distribution is discontinuous at first, but on standing overnight it becomes more nearly linear (this may be speeded up by judicious mixing). The virus solution is layered at the top, and the tubes are centrifuged. Each component of the solution then moves down the sucrose gradient as a thin layer. The velocity at which these layers sediment is a variable, because several factors are involved. The centrifugal field increases with the radius (about 2-fold), but the viscosity of the fluid increases about 6-fold from the top to the bottom and, of course, the density difference between the virus and the solution decreases by a factor of about 0.5. The last two effects tend to slow down the boundaries as they descend.

After the required centrifugation time has elapsed, the centrifuge is stopped and the tubes sampled. In some cases, as with turnip yellow mosaic, the zones are clearly visible, but in other cases samples are taken at definite intervals. (If tubes transparent to ultraviolet light are used a concentration of virus of 10  $\mu\text{g./ml.}$  should easily be visible by photography.)

An improvement on the method has been described by Sinclair and associates (1957), who used an apparatus (designed by Prof. R. M. Bock) which produces a linear concentration gradient automatically by mixing the contents of two hypodermic syringes; they used the method to separate the two components of the wild cucumber mosaic virus. In a case like this, where the two components have rather differing specific volumes, the effects of the density of the sucrose solution on the actual separation is not nearly so marked as when the substances to be separated have similar specific volumes.

Ethanol and methanol solutions also present the possibilities of constructing gradients, but the density differential over acceptable concentrations (0–20 %) is only about 0.025–0.03, as opposed to 0.15 for sucrose. Another possibility is the use of inorganic salt solutions, such as solutions of the halides of the alkali metals, where densities of the same order as those obtainable with sucrose can be obtained but with lower viscosities. Such solutions will not usually precipitate viruses, but they may damage some of the less stable ones.

The density gradient method has been used with some success for the purification of some of the larger and not very stable viruses, such as the wound tumor virus (Brakke *et al.*, 1954). This method, combined with the use of radioactive isotopes, might well yield some interesting information about these large viruses which are capable of multiplication in insects as well as in plants. A point of particular interest in this regard is whether they contain ribonucleic acid like other plant viruses, or if, like many of the large viruses of animals, they contain deoxyribonucleic acid.

#### *E. Following the Course of a Purification*

The course of purification of a plant virus is best followed by activity measurements. These are not altogether reliable, but the first thing which



characterizes a virus and differentiates it from nonviral material is its ability to cause disease. Unfortunately, the most rapid of infectivity tests takes at least 24 hours and then only under exceptional circumstances. Local lesions usually take 3 days to develop, while systemic symptoms take a week or more. Consequently, other methods are used whenever possible. Of these, probably the most useful one is the ultracentrifugal examination. Most viruses can be followed by this means after the first stage of concentration has been passed, and usually viruses have a characteristic sedimentation rate and very sharp boundaries. Such a test takes less than an hour.

Spectrophotometry is also a very useful test. Most plant viruses absorb with a maximum at about  $260\text{ m}\mu$  and a minimum at about  $240\text{ m}\mu$ . The extinction at the peak for virus solutions of  $0.1\text{ mg./ml.}$  is usually between 0.2 and 0.8. Impurities usually remove the trough in the  $240\text{ m}\mu$  region. Pure virus usually has very little absorption at  $320\text{ m}\mu$ , but when the larger plant viruses come to be investigated appreciable absorption can be expected in this region because of their scattering potentialities.

Serological testing is also of considerable use in following a purification, but the results may be complicated by the presence of noninfectious antigens characteristic of the virus infection. As an example, if one were to follow the purification of the Rothamsted strain of the tobacco necrosis virus (q.v.) by infectivity measurements and by serology, one might end with two different substances.

### *1. Infectivity measurements*

Most infectivity measurements are of a low precision, and consequently it is of little use to make dilutions at finer intervals than 10-fold. Comparisons are made on opposite halves of leaves, because the leaves of a batch of plants may vary a 100-fold in susceptibility. It is also important that the inoculations of control and experimental dilutions should be made more or less simultaneously because plants show a diurnal change in susceptibility, which is much greater than one might imagine.

Provided that the number of lesions per half leaf is not excessive, preferably less than one hundred, there is a roughly linear relationship between the logarithm of the local lesion count against the logarithm of the weight of virus per milliliter which may be used for interpolation. Usually this is not warranted unless large numbers of plants are used.

With plants which do not give local lesions the only possible solution is to inoculate batches of 10 or 20 plants with each dilution. Because of the variation between the plants this method is very inaccurate and not to be compared with the apparently similar titration of viable organisms using 10-fold dilutions and inoculating several samples of each dilution.

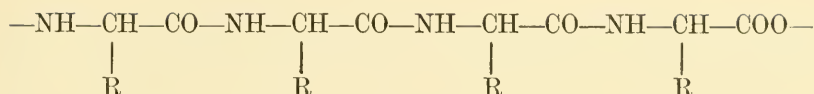
### III. THE PROTEIN COMPONENTS OF PLANT VIRUSES

As has already been indicated, the greater part, amounting in some cases to over 90 %, of the weight of virus particles consists of protein. Up to a few years ago proteins were known to have very remarkable properties, which had been determined from the study of enzymes, hormones, blood pigments, and other biologically active substances having a protein nature, and were regarded as the quintessence of life itself. In fact, according to one eminent authority, life is "the mode of existence of the proteins." Recently, of course, the recognition of the importance of nucleic acid in living processes has resulted in a decrease of the popularity of the proteins, and, in all probability, the pendulum may have swung too much in the other direction. The very fact that the viruses, which are among the smallest self-perpetuating systems known, invariably are associated with protein, and that of a highly specific type, suggests that protein plays an important part in their economy. Fortunately the study of virus proteins has not been neglected, because it holds out the chance, slim as it may seem, that, in the study of the two major components of the viruses, one might possibly disentangle the mysterious but fundamental code relating the structure of nucleic acids to that of the proteins. At the present moment, the prospects of a successful conclusion to this work are not as happy as one might hope, because the functions of the two major components of the plant viruses are by no means fully understood, but there is little doubt that some important discoveries will result from this type of research.

What then is known about the structure of proteins in general, and of the virus proteins in particular? All proteins consist in the main of  $\alpha$ -amino acids, compounds having the structure  $R-CHNH_2-COOH$ , where R is a residue, or side chain, and usually joined to the remainder of the amino acid molecule in such a way as to make the  $\alpha$ -carbon atom levorotatory. In the simplest amino acid, glycine or  $\alpha$ -aminoacetic acid, of course, R is a hydrogen atom and then the possibility of optical isomerism does not exist. In the other cases the R-groups are all characteristic, and in normal proteins some 20 are to be found, although in certain proteins only 16 or 17 of the possible amino acids may be present, while in others there may be amino acids of a quite unique structure. It will be appreciated that the possible number of R-groups is to all intents unlimited, and it is a point of interest that, by and large, the known organisms can make do with only 20 of the amino acids for making their proteins.

#### *A. Polypeptides*

The amino acids are joined together as the amides of the adjacent carboxyl groups in the chain, by what are called *peptide* links. Thus a polypeptide chain has the general structure:



It will be noted from this that, in general, one end of a polypeptide chain has a free amino group, while the other has a carboxyl group, so that the molecule has both acidic and basic properties, irrespective of the nature of the R-groups, and that the latter are to one side of the chain proper. Thus the peptide linkages are primarily structural, while it is largely the nature and arrangement of the side chains which give the peptide chains and the proteins their distinctive properties.

The proteins themselves consist of vast polypeptide chains, not arranged in a linear array as depicted above, but probably intricately coiled and folded and, in the case of the virus proteins, assembled into compact lumps containing perhaps as many as 200 or more amino acid residues. Each virus, of course, is made up from many of such lumps or subunits, the total number of amino acid residues in a virus like tobacco mosaic virus being of the order of half a million.

The present view of the way in which the polypeptide chains are folded is that proposed by Pauling and Corey (1952), and known as the  $\alpha$ -helix. This structure has one or two features of special interest. The amino acid residues are arranged in a tight helix with 3.7 residues per turn, resulting in a fairly compact central region, consisting mainly of the polypeptide backbone, while the side chains are arranged on the outside of the helix, and come off at an angle to the main axis. This has the effect of making the relative positions of the ends of the side chains quite different from the relative positions of their  $\alpha$ -carbon atoms. This makes the determination of the surface structure of a polypeptide a matter of extreme difficulty, even if the amino acid sequence is perfectly well known, and as it is almost certainly the surface structure with its intricate arrangement of various hydrophylic, hydrophobic, and reactive groupings which decides the biological activity of the protein, it will be realized that the problems which lie ahead are extremely complex.

A structural feature, which may explain the presence of the one imino acid (proline) in polypeptide chains, is that its structure causes an interruption in the sequence of peptide linkages, and so allows a kinking or folding to take place between  $\alpha$ -helix sections. Cross linkages can be made between adjacent helices by the  $\text{—CH}_2\text{—S—S—CH}_2\text{—}$  bridges of cystine, thus stabilizing the final structure, and several other cross links are possible.

### *B. The Determination of the Composition of Proteins*

Before the advent of chromatographic methods for the detection and estimation of amino acids, the problems involved in the analysis of viruses

were such as to deter all but the most optimistic workers. It is true that colorimetric methods existed for the estimation for a number of amino acids, such as tryptophan, arginine, and tyrosine. Total sulfur analysis, of course, would give the cystine plus methionine, and so on. Microbiological assay, using microorganisms having fastidious requirements for amino acids, have been employed with some success, but these depend upon the measurement of growth of the microorganism or the production of acid from carbohydrate as a criterion of growth, and such methods are fraught with the dangers of the production of growth inhibitors and/or stimulants during the hydrolysis of the protein in order to produce the free amino acids for the assay. In addition, the techniques for the hydrolysis of the proteins did not give quantitative yields of the amino acids, particularly in the presence of nucleic acid, which, apart from yielding glycine as a breakdown product from the purine rings (Markham and J. D. Smith, 1949), may interfere with the recovery of some amino acids because of its sugar content.

In recent years, however, the techniques of amino acid analysis have been greatly refined. Qualitative analysis may be performed either by paper chromatography or by paper electrophoresis, the latter requiring as little as half an hour's time to complete, and the ion exchange procedures developed by Moore and Stein (1951, 1954) are now enabling routine analyses to be made on a few milligrams of total material—analyses which have adequate precision for all normal purposes, and which can now be made by semi-automatic apparatus.

The analyses which have been performed so far on the plant viruses have been more or less tentative, looking for the presence or absence of the common amino acids, and attempting to find any striking peculiarities which make the virus proteins stand out from other proteins. So far such studies have been interesting, but are far from throwing much light on the way in which viruses accomplish their object. What has been found is that similar viruses may have very closely similar general amino acid compositions. It is quite evident, however, that the mere routine analysis of the amino acid contents of various viruses is not going to yield as much useful information as would determination of the amino acid sequences in the virus as a whole. Now as there are, as has already been mentioned, some hundreds of thousands of amino acid residues in a single small plant virus, this task might *a priori* seem to be an absurdly difficult one to undertake. However, it has been realized for many years, largely owing to the work of Bernal and Fankuchen (1941a,b), who were studying the structure of viruses with X-ray diffraction, that the tobacco mosaic virus was built up of many similar, if not identical, subunits, which were of the same order of size as small protein molecules. The significance of this work was very largely overlooked until a few years ago, when Harris and Knight first investigated the action of carboxypeptidase



on this same virus. Carboxypeptidase is an enzyme having *exo*peptidase activity, that is to say, it attacks the terminal amino acids in a peptide chain in sequence, and this particular enzyme attacks the end of the chain which has a free  $\text{—COOH}$  group. Rather to their surprise, Harris and Knight (1955) found that only threonine was liberated, and in fact this amounted to some 2900 residues per 50,000,000 molecular weight or, if the somewhat lower estimate of molecular weight favored by some authorities is taken, some 2300–2400 residues of this one amino acid are liberated. Assuming that each subunit of the virus yields one and only one residue of threonine, this gives a subunit size of some 17,000 molecular weight, a size which although large is still within the range which one might visualize tackling by the methods either available at present or being developed. Moreover, this size agrees well with the values estimated from the cysteine content of the virus protein ( $M = 18,000$ ). The main difficulty which arises is that one has to assume that the virus is made up from only one type of subunit and that all these subunits are identical. That this is an unlikely assumption is, of course, obvious because the isotopic distribution of the atoms constituting the structure precludes absolute identity. But if one ignores this obvious limitation, what are the chances that the subunits are closely similar? In fact, it might appear extremely likely that the polypeptide subunits of a virus should be limited in variety; it is not unlikely that the subunits may be of one type only, and, if one considers that the only obvious function of the protein part of, say, tobacco mosaic virus is protective, it is likely that they are. Furthermore, as has been noted, the chemical evidence to date indicates that this is so, as well as do the X-ray diffraction data. However, it is not unthinkable that minor irregularities do exist. In fact, it would be surprising if they did not. Also, it is possible that the very ends of the virus rods may be different in some degree from the rest, although the great tendency of the virus to aggregate in a linear direction might suggest the opposite.

There is, of course, a certain indication that the virus rods are in fact not uniform. For example, Harrington and Schachman (1956), studying the degradation of tobacco mosaic virus by alkali, found that a small proportion of particles in any one preparation are much more resistant to the action of the alkali than are the remaining particles. Furthermore, they found that the alkaline degradation of the particles was more rapid at the beginning, and that when the particles had been degraded to about one-third of their original length, the remainder was much more resistant to the chemical action. This latter observation is, of course, by no means conclusive evidence of the essential difference of the remaining fragment of the rod, but it is suggestive. However, if one were to have to give a dispassionate summing-up of the present situation, it would be that there is a chance that by making the

possibly oversimplified assumption that all subunits are identical or closely similar, one might find out interesting and, possibly, important facts. The reader should, however, be referred at this stage to a review by Pirie (1956b) relevant to this matter.

Following upon the work on tobacco mosaic virus, Crick and Watson (1956) pointed out that the most economical and rational way of constructing a "spherical" virus was also from subunits, all of one kind fitted together in an array which was based upon the geometry of the regular solids, and the model which they favored was the solid figure, the icosahedron (Kaesberg, 1956), which has 20 equilateral triangular faces. Placing 3 subunits, which may, of course, have their own substructure, in every equilateral triangle, one then has a solid figure having 60 identical subunits. This gives a polypeptide subunit having a molecular weight of about 100,000, a value which is reasonable, and also gives a structure which is more or less compatible with the known features of some of the smaller "spherical" viruses. It should be remarked here that this type of regular figure is likely to be associated with those viruses which crystallize in a cubic or hexagonal lattice, or an approximation to the latter. There are, of course, viruses such as the southern bean mosaic virus, which have shapes incompatible with this concept of high symmetry, while the very recent observations of the alfalfa mosaic virus (Bancroft and Kaesberg, 1958) are even less compatible with this type of model. However, for those viruses, such as the turnip yellow mosaic and bushy stunt viruses, which show a high degree of symmetry, it is not unlikely that they possess a regular subunit which is one-sixtieth of the whole, and which itself may consist of a number of subunits. The latter may, of course, not all be identical, but the whole structure, if made up in this way, would be susceptible to investigation by orthodox chemical methods with some possibility of success. Whatever transpires, it is certain that the next few years will yield exciting results.

### *C. Methods for the Determination of the Structure of Polypeptide Chains*

The methods which are at present available for studies of this kind are essentially of three types. These are:

#### *1. Determination of Amino Acid Composition*

Given a pure peptide, the determination of its amino acid composition is easily performed by chromatographic methods. If it is a small polypeptide containing, say, 4 residues, simple chromatography on paper will suffice. If it is very large, the Moore and Stein column chromatography will have to be applied, bearing in mind the loss or destruction of such amino acids as cystine and tryptophan during hydrolysis.



## 2. Determination of the Nature of the Chain Ends

a. *N-Terminal Residues.* The N-terminal amino acid (that having a free  $\text{—NH}_2$  group) may be identified by one of a number of methods. That most suitable for general use is the reaction of the peptide with 1,2,4-fluoro-dinitrobenzene, which may be effected in aqueous solution near neutrality (Sanger, 1945). The N-terminal amino acid residue is converted to a dinitrophenyl amino acid residue, which may be identified after hydrolysis, particularly as most dinitrophenyl amino acids are yellow in color and most are soluble in ether. It is, of course, necessary to take account of the  $\epsilon$ -dinitrophenyl derivative of lysine, which reacts even when not terminal. The reagent may also substitute some groupings such as  $\text{—SH}$  which do not contain nitrogen.

Other methods which may be used are the acetylation of the free  $\text{—NH}_2$  groups, for example, by ketene, or the deamination of the former by  $\text{HNO}_2$ , when they yield  $\alpha$ -hydroxy acid residues.

b. *C-Terminal Residues.* The C-terminal residue, or that bearing a free  $\alpha$ -carboxylic acid group, can also be identified in a number of ways. The simplest is the hydrazinolysis method of Akabori *et al.* (1952), in which the peptide is treated with anhydrous hydrazine. During the reaction all the carboxyl groups involved in peptide linkages are converted to hydrazides, while the C-terminal amino acid is released unchanged.

## 3. Determination of Sequence

The determination of the amino acid sequence is, of course, one of the ultimate ends in the determination of the structure of a protein. Its accomplishment is a matter of extreme difficulty, increasing as a power of the number of amino acid residues in the protein. While theoretically it is possible to degrade a protein from one end, removing amino acid residues successively, practical considerations rule this out, and the practical approach is as follows:

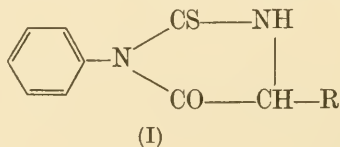
a. The protein must be hydrolyzed, preferably in more than one way, to give a number of small polypeptides. This may be accomplished by chemical hydrolysis, with acid or alkali, or by enzymes. In this hydrolysis care must be taken to avoid the possibility of the synthesis of structures not already present in the protein. The hydrolysis of polypeptide chains into easily recognizable fragments is a matter of considerable difficulty. It can be shown that any method which does not have absolute specificity, or at least a very high degree of specificity for a particular set of bonds, is not likely to be of much use for a large polypeptide. This more or less excludes the use of acid- or base-catalyzed hydrolysis for most work, and one is forced to use enzymatic hydrolysis, with the inevitable possibility of synthesis concomitant with degradation. Also there are few enzymes of the endopeptidase

type which show a high degree of specificity or, at least, a specificity which is fully understood. Outstanding, of course, is trypsin which breaks only arginine and lysine peptide bonds. Thus, a polypeptide which had, say, 10 arginine and 3 lysine residues would be broken into 14 fragments, 10 containing a terminal arginine residue and 3 having a terminal lysine residue, the basic amino acids being at the  $\text{—COOH}$  termination of the chains. The last fragment will contain neither arginine nor lysine. Fragments containing arginine can, of course, easily be detected by the specific Sakaguchi reaction. Furthermore, the enzyme may be induced to split only the arginine-containing linkages by the simple procedure of blocking the  $\epsilon$ -amino groups of the lysine residues by carbobenzoxy groups which may later be removed by means of HBr (Anfinsen *et al.*, 1956). This simple trick has not been exploited to the full, but of course will give some overlapping regions. Also, it should be possible to regenerate the lysine residues and then apply trypsin a second time.

$\alpha$ -Chymotrypsin, which is also much used, has a specificity which is as yet not fully defined, but it does give rise to a number of recognizable fragments. Pepsin, which has also been used, is less promising in general. Its splitting activity tends to be toward the aromatic amino acid linkages, but is by no means specific for these only. It goes without saying that the enzymes used for this type of work have to have an extremely high degree of purity. This is often difficult to obtain.

b. Having been formed, the peptides must then be separated in a relatively pure form from each other. This is the step which involves most of the labor. Techniques used may involve electrophoresis, paper chromatography, ion exchange chromatography or even countercurrent distribution, although the latter has relatively low resolution and is extremely time-consuming.

c. The individual small polypeptides must then be analyzed. For di- and tripeptides the procedure is, of course, relatively simple, but for the larger ones recourse must be had to more specialized techniques. One of the most promising is the Edman (1950) degradation, which involves the successive treatment of the polypeptide with phenyl isothiocyanate which reacts with the free  $\alpha$ -amino group to give a phenyl isothiocarbamyl amino group. On treatment with anhydrous HCl the terminal amino acid residue is split off as a phenyl thiohydantoin which absorbs ultraviolet light and which may



be identified, leaving the remainder of the polypeptide intact. Thus this

method is suitable for the stepwise degradation of polypeptides, and it may in fact be applied to very small amounts of material.

Carboxypeptidase may also be used for the sequential splitting-off of amino acids, this time from the C-terminal end. Unfortunately, the rate at which the individual amino acids are split off depends, to a large extent, on the nature of the amino acid next in sequence in the chain, so that it is necessary to determine the rates at which the successive amino acids are released; even then it may not be possible to identify more than the first one or two in each sequence. In the case mentioned earlier of the intact tobacco mosaic virus, only the amino acid, threonine, is liberated, because the succeeding residues are alanine followed by proline, and the prolyl peptide bond is completely resistant to attack by this enzyme.

Finally, having determined the sequences of a large number of peptides, the polypeptide sequence may then be determined *provided* that enough overlap of sequence exists to enable the pieces to be fitted together, like a jigsaw puzzle, in an unambiguous way. Needless to say this has not yet been accomplished for any virus.

#### IV. THE NUCLEIC ACIDS

The nucleic acids were first recognized in animal cell nuclei in the latter part of the last century by Miescher (1871) who obtained a phosphorus-rich compound, which was evidently acidic, and which he realized was one of the important organic phosphorus compounds of animal tissues. Substances of this general type were later isolated from plant cells (Altmann, 1889; Osborne and Harris, 1902), but the general impression of the early workers was that animal tissues contained one type of nucleic acid, and plant tissues another. There are, indeed, two main types of nucleic acid, which differ mainly in their sugar constituents, but most cells of animals and plants contain both. Viruses usually contain one or the other, and plant viruses, as far as is known at present, contain only that nucleic acid which has ribose as its sugar, or ribonucleic acid, as it is now called. Strictly speaking, ribonucleic acid is a term covering a family of related substances, all having similar structures but differing in very subtle ways. These differences are thought to be related to the biological function of the nucleic acids.

For very many years, the nucleic acids were regarded as relatively simple compounds having molecular weights of the order of 1200; as they were invariably associated with protein, they were regarded rather as an adjunct to proteins, in combination with which they formed the nucleoproteins, than as distinct chemical substances possibly having biological activity of a high order. Indeed the term "prosthetic group" was originally used to denote the relationship between nucleic acid and protein by Kossel (1893).

One of the main reasons why the nucleic acids were not recognized as giant molecules was that their relative instability was not appreciated; another was that the methods for characterizing large molecules had not been sufficiently developed.

It was not until the late 1930's that, largely owing to the insistence of cytologists, the biochemists realized that something was wrong with their ideas about nucleic acids, and this was reinforced later by the discovery of the biological activity of a nucleic acid (this time of the "animal" or deoxy-ribose type) derived from certain strains of pneumococcus by Avery *et al.* in 1944. Shortly after this methods for investigating nucleic acids were more or less revolutionized by the application of chromatographic techniques. It has since become very obvious that the nucleic acids are a group of exceedingly complex molecules, which are usually partially degraded during their isolation and examination, that their general chemical composition is readily amenable to analysis, and, unfortunately, that their detailed structure presents considerable difficulty in its elucidation. Of all nucleic acids, however, those associated with the plant viruses look the most promising for investigation. Indeed, as will be discussed later, there is some indication that the tobacco mosaic virus may contain one unique chain of nucleotides. If this does prove to be so there is a possibility that quite a lot of information about the general structure of the nucleic acid might be obtained. Unfortunately its size is such that the hope of determining fine details of structure is slight.

The nucleic acids, like the proteins, contain a structural part, and a part which confers their specificity. The backbone of the nucleic acids is a chain of sugar residues, either consisting of ribose or deoxyribose, joined by an equivalent number of phosphoric acid residues, and forming a linear polymer, which may, of course, be coiled or folded because it is flexible about the phosphate linkages. The latter join C<sub>5</sub> of one sugar to C<sub>3</sub> of the next one by ester bonds to the primary and secondary alcoholic groups of the sugar which are in these positions.

The biological specificity of the nucleic acid is conferred by the presence of a number of kinds, usually four, of different nitrogenous heterocyclic compounds, which are present in most, if not all, cases as *N*-glycosides. The arrangement and nature of the nitrogenous compounds, or bases, as they are commonly called, is thought to confer the specificity on the molecule, although the mechanism is as yet obscure. It will be noted that four substituents are more than are necessary for conveying information. In fact the molecule is not by any means an economical repository of information, and the presence of at least four substituents may well be occasioned by the mechanisms by which the nucleic acid is synthesized. It is also possible that, in ribonucleic acid at least, the compound fulfils a dual purpose.



In addition to the above, many nucleic acids contain small amounts of methylated purines and pyrimidines. So far these have not been detected in virus nucleic acids. 5-Methylcytosine is not known to occur in viruses (Wyatt, 1951), while 5-hydroxymethylcytosine is peculiar to certain bacteriophages (Wyatt and Cohen, 1953).

Certainly all four usual constituents of ribonucleic acid possess coenzyme activity in one or more systems.

### *A. The General Structure of Nucleic Acids*

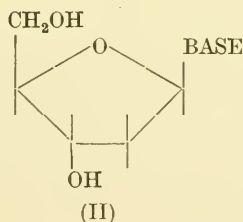
The nucleic acids are divided into two main classes, the ribonucleic acids, which contain D-ribofuranose, and the deoxyribonucleic acids, which contain D-2-deoxyribofuranose. These sugars are in  $\beta$ -N-glycosidic linkage with purines, which form 9-glycosides, and pyrimidines, which form 3-glycosides. The bases commonly found in nucleic acids are shown in Table I.

TABLE I  
BASES ASSOCIATED WITH VARIOUS NUCLEIC ACIDS

| Bases       | Ribonucleic acids  | Deoxyribonucleic acids   |
|-------------|--------------------|--|
| Purines     | Adenine<br>Guanine | Adenine<br>Guanine   |
| Pyrimidines | Cytosine<br>Uracil | Cytosine<br>Thymine<br>5-Methylcytosine<br>5-Hydroxymethylcytosine |

#### *1. Nucleosides*

By suitable degradation methods the sugar glycosides of bases may be liberated. These are called nucleosides, and have the general structure shown in Formula II.



The nucleosides are named for their bases. Thus, the adenine riboside is called adenosine. The other nucleosides are guanosine, cytidine, uridine,

and thymidine. Where ambiguity about the sugar may exist the deoxy-riboside is specified as in deoxyadenosine, the term "adenosine" being taken to imply riboadenosine.

## 2. Nucleotides

The phosphoric esters of the nucleosides are called nucleotides. It will be seen from the structure of ribose and deoxyribose that several nucleotides can exist. Ribose in the furanose form has two secondary alcoholic groups (2 and 3), and one primary alcoholic group (5), and so can form three mono-ester phosphates. In addition two diester phosphates can be formed, namely, the 2 : 3 (cyclic) phosphate and the 3 : 5 (cyclic) phosphate. The latter is purely of academic interest at the present time, as far as ribonucleic acid is concerned.

Deoxyribose nucleosides have only one primary and one secondary alcoholic group, and so only form two monoester phosphates, namely, the nucleoside 5'-phosphate and the nucleoside 3'-phosphate. They can also form 3' : 5' (cyclic) phosphates.

All of these compounds except two may be obtained from nucleic acids during their degradation (the nucleoside 3' : 5'-phosphates have not been encountered so far).

Nucleotides are usually named after their bases, for example, adenylic acid, deoxycytidylic acid, and so on. This nomenclature is ambiguous because of the possibility of several nucleotides existing, so that if the nucleotide has to be specified more precisely, the nucleoside and its substituent are both specified, as in deoxyguanosine 3'-phosphate.

## B. The Ribonucleic Acids

The nucleic acids which have been found in all those plant viruses which have been analyzed so far are ribonucleic acids. The fact that the sugar is ribose has been shown several times, usually by paper chromatography, which effectively separates all the pentoses. No unusual bases have been found, so that the only nucleotides encountered have been adenylic acid, guanylic acid, cytidylic acid, and uridylic acid. These nucleotides may be liberated by enzymatic or chemical hydrolysis, and then may be analyzed by ion exchange chromatography, or more rapidly on paper. Then the relative quantities of the four compounds may be estimated by means of their ultraviolet absorption (the molar extinction coefficients are of the order of  $10^4$ ). Alternatively, the nucleic acids may be hydrolyzed to give adenine, guanine, cytidylic acid, and uridylic acid (as well as ribose and phosphate, which are not estimated) which may be separated readily on paper chromatograms. Such analyses may be made on much less than one milligram of



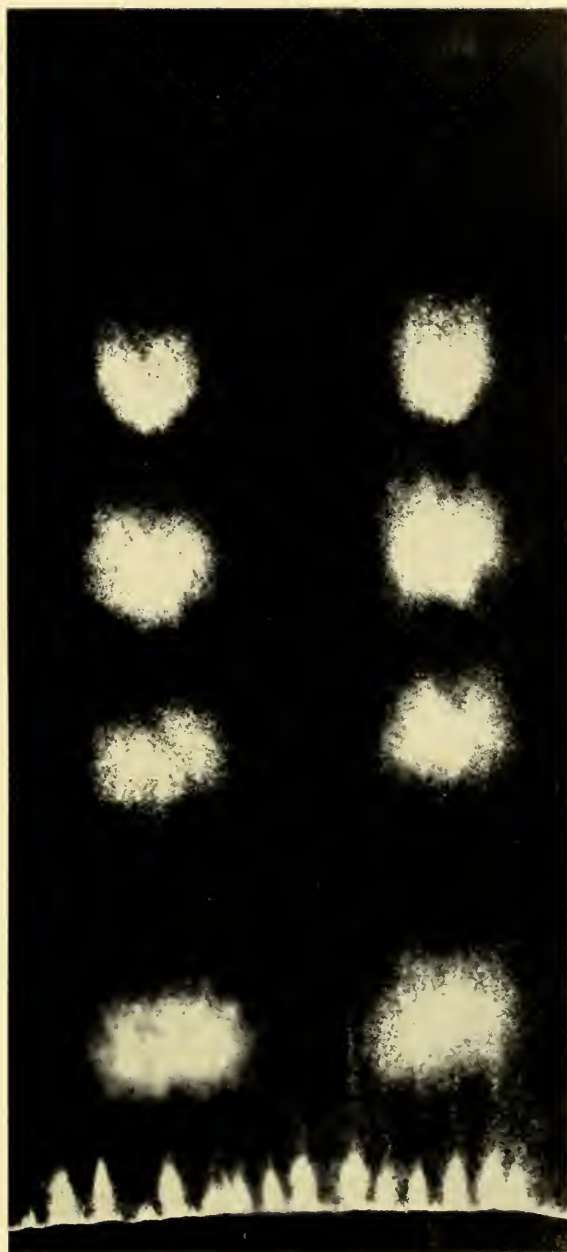


FIG. 3. A paper chromatogram of a hydrolyzate of the nucleic acid from a strain of tobacco mosaic virus, photographed in ultraviolet light of  $265\text{ m}\mu$ . The spots from the top are: guanine, adenine, cytidylic acid, and uridylic acid. Duplicate specimens were run side by side.



virus nucleic acid with reasonable precision (Fig. 3) (J. D. Smith and Markham, 1950; Markham and J. D. Smith, 1951b). For the analysis it is unnecessary to remove the protein, because ribonucleic acids hydrolyze much more readily than does protein, and in any case the absorption of the protein constituents in the ultraviolet is quite minor compared with that of nucleotides or of purines.

Another method of hydrolysis which has been used for ribonucleic acids is by means of strong (about 11 *N*)  $\text{HClO}_4$  (Marshak and Vogel, 1950). This method has much to commend it for the analysis of small polynucleotides, but it is not reliable enough for accurate analysis.

The analyses performed on the plant viruses and summarized in Table II

TABLE II  
THE COMPOSITION OF SOME PLANT VIRUS RIBONUCLEIC ACIDS<sup>a</sup>

| Virus                | Adenylic acid | Guanylic acid | Cytidylic acid | Uridylic acid |
|----------------------|---------------|---------------|----------------|---------------|
| Tobacco mosaic       | 1.20          | 1.01          | 0.74           | 1.06          |
| Cucumber 3 and 4     | 1.03          | 1.03          | 0.75           | 1.21          |
| Turnip yellow mosaic | 0.92          | 0.68          | 1.52           | 0.88          |
| Tomato bushy stunt   | 1.00          | 0.02          | 0.88           | 1.00          |
| Potato X             | 1.37          | 0.87          | 0.91           | 0.85          |
| Tobacco necrosis     | 1.12          | 0.98          | 0.88           | 1.03          |
| Southern bean mosaic | 1.03          | 1.04          | 0.92           | 1.01          |
| Turnip crinkle       | 1.10          | 1.02          | 0.98           | 0.89          |

<sup>a</sup> The above figures are obtained from Knight (1954) and Markham and J. D. Smith (1954), which give data obtained from several sources. The values are rounded off; in general, the two sources referred to agree to within 5 %. The data on turnip crinkle virus are merely included for interest.

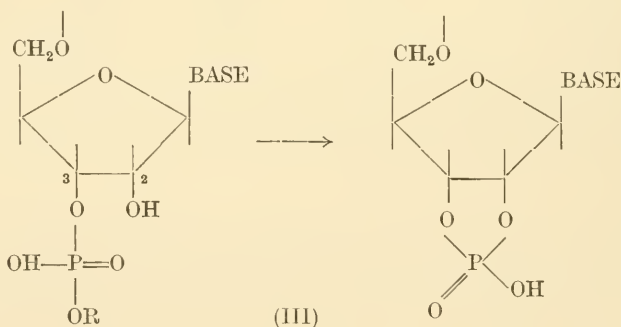
have shown that the ribonucleic acids do not have equivalent amounts of the four bases and also that, in general, related viruses have very similar general nucleic acid compositions. Of course, as these substances are very complex, it is not possible on the basis of estimating their nucleotide (or base) ratios to decide whether two viruses are related. Indeed, there is at least one instance known to the writer where this was not the case, and, all in all, the simple estimation of four substances, all present in about equal amounts, is not likely to give more than a small amount of information about the composition of substances as complex as nucleic acids. In fact it is surprising that the differences found are so great.

### *C. The Finer Structure of the Ribonucleic Acids*

The types of bonds present in the ribonucleic acids which link the various nucleotides together are now fairly well recognized. The determination of

these linkages presented quite considerable difficulty in the first place, however, because it had to be accomplished by degradation methods of uncertain specificity.

One of the main peculiarities of ribonucleic acids is their extreme lability to the action of alkali. Phosphate diesters are usually very stable to the action of alkali, but ribonucleic acids are converted quantitatively to mononucleotides by *N*-KOH at 20°C. for 18 hours. Originally these mononucleotides were considered to be nucleoside 3'-phosphates (largely because the latter are less soluble than the corresponding 2'-phosphates and so are more readily precipitated), but work done by Cohn (1950) and Carter (1950) showed that the adenylic nucleotides were in fact two in number, and it was later found that all the mononucleotides in the alkaline digest occurred in pairs. The nature of these nucleotides and the reason for the lability of ribonucleic acids were eventually clarified in an interesting theoretical discussion by Brown and Todd (1952). Arguing from the known behavior of other phosphate diesters, they showed that, if in ribonucleic acid the phosphate was linked to C-2' or C-3', and esterified elsewhere as well, the presence of the adjacent hydroxyl group at C-3' or C-2' would aid the degradation. The phosphate first esterifies the adjacent hydroxyl group which is in the *cis* relationship, so forming a nucleoside 2' : 3'-cyclic phosphate, as shown in Formula III.



The latter is then hydrolyzed randomly to give either the nucleoside 2'- or 3'-phosphate. (It will be noted that the first stage in the degradation is *not* hydrolytic but a transesterification. The second, and slower, step is hydrolytic.)

Brown and Todd were of the opinion that the cyclic nucleotide would only be a transient intermediate, but Markham and J. D. Smith (1952a) showed that it could be isolated from alkaline digests, and also that compounds of this type were intermediates in the digestion of the nucleic acid by pancreatic ribonuclease, which attacks them relatively slowly.

These observations more or less established that the phosphate ester link was through C-3' or C-2'. The unfortunate migration of the phosphate, of course, precluded the decision whether C-2' or C-3' was the actual original site of the ester linkage, but this was later clarified by Brown and Todd (1953) for the pyrimidine nucleotides by using model compounds to deduce the absolute substrate specificity of pancreatic ribonuclease, which they found to attack pyrimidine nucleoside 3'-phosphate benzyl esters, but not the analogous 2'-compounds. As far as the purine nucleotides were concerned, they were shown to have phosphate at C-3' by two independent methods. Whitfield and Markham (1953) used a chemical degradation method on dinucleotides, and prepared the purine nucleoside 3'-phosphates. Heppel and associates (1953) degraded dinucleotides using a diesterase from spleen, which does not give a 2' : 3'-cyclic nucleotide intermediate, and they also obtained nucleoside 3'-phosphates from polynucleotides by this route.

The other end of the linkage was determined in several ways. Cohn and Volkin (1952) obtained nucleoside 5'-phosphates from ribonucleic acids by enzymatic degradation. This method is now in general use for analytical purposes. Another piece of evidence was produced by Markham and J. D. Smith (1951a, 1952b), who showed that some dinucleotides, produced by ribonuclease degradation from virus ribonucleic acids, had a terminal nucleoside 2' : 3'-cyclic phosphate residue, and hence, by elimination, had to have their C-5' involved in the internucleotide linkages.

The further structural study of virus ribonucleic acids has been based very largely on the use of pancreatic ribonuclease. This enzyme is specific for pyrimidine nucleoside 3'-phosphate esters, and so will break the polynucleotide chain in places where these compounds are present. In consequence, one gets a series of products having the general formula:  $n$  purine nucleotides + 1 pyrimidine nucleotide. The factor  $n$  may have any value from 0 upward. Markham and J. D. Smith (1952b) isolated and identified a large number of such products from digests of the turnip yellow mosaic virus, some of them incomplete digestion products. But, as has been shown by Heppel *et al.* (1955), the enzyme has considerable synthetic potentialities, so that data obtained from partial degradation are not unequivocal. Provided, however, that the system is free from other enzymes, the final products are unique. Unfortunately it is by no means certain that any nucleic acid has yet been obtained completely free from degradative enzymes, and so the results of enzymatic degradation must be regarded with a little reserve. There is, of course, not much doubt that the bulk of the data is in fact relevant, but with very large molecules a little impurity may give rise to artifacts, which may be difficult to detect.

Most other enzymes have even less specificity than pancreatic ribonuclease, and are only really suited to the attack on small polynucleotides.



Methods for studying such molecules by means of enzymes are listed in the above reference (Heppel *et al.*, 1955).

The chemical degradation of polynucleotides is even more difficult, but may be made to yield dinucleotides (Merrifield and Woolley, 1952; Cohn and Markham, 1956), but only in small yield. A promising method is that of Whitfeld (1954), which is essentially a procedure for stepwise degradation. Unfortunately it too has to rely on an enzymatic step in order to produce a polynucleotide chain ending in a nucleoside residue with free —OH groups at C-2' and C-3'. The enzyme used, human prostate phosphatase, can be prepared essentially free from detectable nucleases, but the possibility of nuclease action on a long chain is not predictable with absolute certainty. Having removed the terminal phosphate residue, the nucleic acid is then subjected to the action of sodium periodate. This reagent, which is remarkably specific, oxidizes the *cis*-glycol at the terminal nucleoside residue to a dialdehyde. Such a compound has a —C=O group in the  $\beta$ -position in relation to the phosphate ester linkage at C-5', and the latter is thus rendered very labile. On exposure to a pH of 10, the phosphate group (and the rest of the chain) undergoes elimination. The net effect of this reaction is that the residue of the terminal nucleotide drops off the chain, and leaves a chain reduced in length at one end by one nucleotide residue. The phosphatase treatment may then be repeated, followed by the oxidation, and so on. This procedure has been followed quite successfully on small polynucleotides. Of course, for a ribonucleic acid with a chain length comparable with that of, say, the tobacco mosaic virus nucleic acid, an absolute minimum of 100 mg. of material would be needed for the procedure, and the manipulations become exceedingly complex. In addition the tobacco mosaic virus nucleic acid has sufficient plant ribonuclease attached to it to break the chain spontaneously, so that the results to be expected are not too hopeful.

Another method for determining the ends of ribonucleic acid chains is dependent upon the specificity of pancreatic ribonuclease. Because of this specificity any purine nucleotides, either 2', 3', or 2' : 3' cyclic ones, which may be liberated, or any small polynucleotide terminated by such a group must have been at one end of the chain, which, of course, is the same end as that detached by Whitfeld's method. These groups are, in fact, readily recognized (Markham and J. D. Smith, 1952c), but their quantity and their distribution are such that they must be artifacts, or else the ends of the nucleic acids must be extremely varied. The former would appear to be the more likely.

A modification of the above treatment is removal of the terminal phosphate groupings by means of phosphatase, followed by alkaline hydrolysis. Any nucleoside residues liberated must represent chain ends.

One method which is free from the objections mentioned above is that due



to Lipkin and Talbert (1955). In this method, which has not yet been fully exploited, the *whole* virus may be dried, dissolved in anhydrous formamide, and then exposed to the action of potassium methoxide. After the reaction is complete, the reactants are removed, still in the absence of water; the resultant mixture contains nucleotide methyl esters, and any nucleotides which are liberated as such must have originated as chain termini. The method is exceedingly exacting, but, in the presence of internal dehydrating agents, should give quite unequivocal results.

The possible kinds of linear polynucleotides are shown diagrammatically in Table III, which shows a chain of 4 (arbitrary) nucleotides having the

TABLE III  
TYPES OF POLYNUCLEOTIDE CHAIN <sup>a</sup>

| Type | Polynucleotide chain |
|------|----------------------|
| 1    | A—p—B—p—C—p—D—p      |
| 2    | A—p—B—p—C—p—D        |
| 3    | p—A—p—B—p—C—p—D      |
| 4    | p—A—p—B—p—C—p—D—p    |
| 5    | A—p—B—p—C—p—D—p!     |
| 6    | p—A—p—B—p—C—p—D—p!   |

<sup>a</sup> A, B, C and D are nucleoside residues. The phosphate residues joining 3'—5' in adjacent nucleoside residues are denoted by p, 3'—5' being from left to right. The exclamation mark denotes a 2' : 3' cyclic phosphate residue.

6 different kinds of ends that are possible. Of these, type 1 can have its right hand end detected after dephosphorylation, or if C happens to be a pyrimidine nucleoside and D a purine nucleoside. Type 2 is type 1 dephosphorylated (or types 3 and 4 dephosphorylated), and the terminal D residue is liberated by alkaline hydrolysis. On alkaline hydrolysis, type 3 gives p—A—p, which is a nucleoside diphosphate, and D, which is a nucleoside. Under the same conditions type 4 will give p—A—p as a detectable end, as will type 6. Type 5 can be treated with ribonuclease if C is a pyrimidine nucleoside and D a purine nucleoside, when D—p! is liberated. Actually, cyclic phosphate ends may be hydrolyzed specifically if necessary, and the chains 5 and 6 converted to types 1 and 4. Chain ends detectable on methanolysis are the right ends of 1, 2, 3, and 4 and the left ends of 3, 4, and 6, which would become monomethyl esters of p—A—p.

At one time it was thought that the tobacco mosaic virus had ends of the type 3 (Markham *et al.*, 1954). These ends were certainly detected, but were probably representative of an external small polynucleotide contaminant. The relative amounts of the ends were relatively large, being about 1 to every 50 residues. If, as is now thought, the nucleic acid chain is continuous and single, the number of ends would be about 1 in every 8000, a

quantity which should be detectable in virus labeled with radioactive phosphate (Reddi and Knight, 1957; Matthews and Smith, 1957).

#### *D. Deoxyribonucleic Acids*

The deoxyribonucleic acids, which differ from the ribonucleic acids by not having an —OH group at C-2' of the nucleoside residues, are in consequence of this stable to alkali and also to ribonuclease. On the other hand, the purine glycosidic link is extremely unstable to acid, and splitting of this link gives rise to an aldehydic function at C-1', which is followed by  $\beta$ -elimination at C-3'. The products of acid treatment (60°C., 0.1 N-HCl, 10 min. or more) are thus chains of the type  $p-nA-p$ , where  $n$  is an integral number, and A any pyrimidine nucleoside (or mixture of the same). The determination of the structure of the small polynucleotides is quite easily accomplished using specific enzymes.

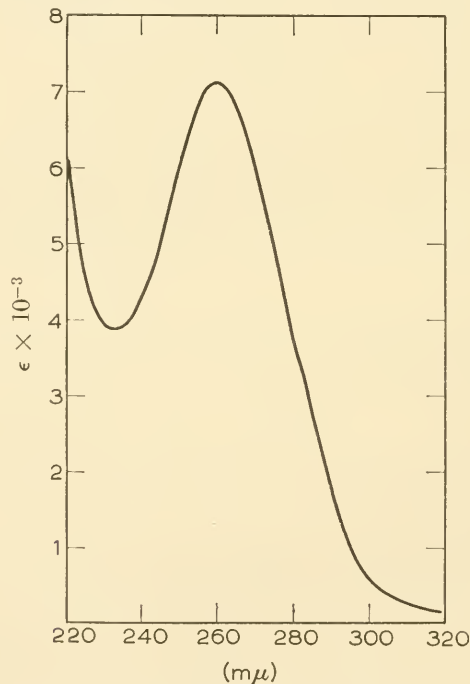


FIG. 4. Absorption spectrum of a typical ribonucleic acid, at 20°C. in water at pH 7.

#### *E. Physical Properties of the Nucleic Acids*

The most spectacular property of the nucleic acids is their extremely high and specific ultraviolet light absorption, which is occasioned by their purine and pyrimidine constituents (Fig. 4). Oddly enough, the light absorption of a nucleic acid is much smaller than that of its constituent nucleotides

on a weight basis, the difference being as much as 30 % for an ordinary polymerized ribonucleic acid. The precise physical meaning of this difference is uncertain, but it is evidently due to intermolecular structure of some kind, such as hydrogen bonding between the absorbing rings. This attribute of nucleic acids makes it very difficult to interpret absorption spectra of substances such as viruses, because the contribution of the nucleic acid to the spectrum is an unknown factor. Based on the moles of nucleotide residues present the molar extinction coefficients of the free nucleic acids in the region of  $260\text{ m}\mu$  are of the order of 7,000 to 11,000 and vary with temperature, but on hydrolysis this rises to some 12,000, the value depending upon the nucleotide composition of the nucleic acid and the pH of the solution.

Being polymers of phosphoric acid esters, which have one primary phosphate  $\text{OH}^-$  per nucleotide residue, and having numbers of  $\text{NH}_2^+$  groups and enolic  $\text{OH}^-$  groups, the nucleic acids are in effect rather like linear ion exchange resins, and have a great affinity for proteins. In fact, the combination of the nucleic acid and the protein of viruses can be explained simply on the assumption of electrovalent linkages existing between the two, although there is evidence that the phosphate groupings are not all involved in such linkages and are to some extent neutralized by basic compounds. The strong affinity of the nucleic acids for proteins is a cause of considerable trouble in their study, because the nucleases, especially the ribonucleases, are extremely stable proteins and are highly basic as well. Consequently when nucleic acids are prepared, they adsorb any traces of nuclease in the solution, and then are slowly digested. It is because of this that while it is relatively easy to make highly polymerized virus ribonucleic acid (Cohen and Stanley, 1942; Northrop and Sinsheimer, 1954), it is extremely difficult to keep it from depolymerizing. The actual estimates for the molecular weights are somewhat dubious. Hopkins and Sinsheimer (1955), using light scattering, estimated the molecular weight of the tobacco mosaic virus to be about 1.7 million, which is rather smaller than the anticipated value of about 2.5 million, if the virus nucleic acid formed one single polynucleotide chain.

Because of their large size and their charge, which tends to make the molecules elongate in water or dilute salt solutions, the nucleic acids form highly viscous solutions or jellies, and show birefringence of flow. Fibers may be drawn both from deoxyribonucleic acids and from ribonucleic acids. The latter are, however, not suitable for detailed X-ray examination, so that little is known about their finer structural details. The synthetic polymers of ribonucleotides have, of course, been examined in this way (Rich, 1957), but it is not altogether certain whether this information is relevant to virus structure.

## V. THE TOBACCO MOSAIC VIRUS

### *A. Introduction*

As has already been mentioned, the tobacco mosaic virus has been investigated more intensively and for a longer time than has any other virus. This is, of course, because it was discovered before any other virus (Iwanowski, 1892), is widespread in nature, is economically important, and above all is extremely infectious and stable (Allard, 1942). In the last two qualities, the tobacco mosaic virus, or, more exactly, group of viruses, is by no means representative of viruses in general. Most plant viruses are far less suitable for laboratory investigation, and it is, indeed, only by the happy circumstances enumerated above in the case of the tobacco mosaic and a few other viruses that our knowledge of these pathogens has advanced as much as it has.

The virus of tobacco mosaic was first isolated in quantity by Stanley (1935) who used a salt-precipitation technique for isolating the infective agent from diseased plant sap. Before this work numerous attempts had been made to purify this virus, a procedure which was largely confused with the removal of plant material rather than the preparation of virus in concentrated suspensions, but there is little reason to doubt that several workers had obtained reasonably pure preparations without realizing it (Brewer *et al.*, 1930; Barton-Wright and McBain, 1933). Apart from the fact that assay of the infective material was not really feasible until the development of the local lesion method (Holmes, 1929; Samuel and Bald, 1933) and that methods for transmitting the infections were so primitive that accidental infection must have been nearly as common as purposeful transmission, the major factor that delayed the recognition of the virus as a substance having definite chemical properties was that nobody expected that it could be present in the quantities which can be obtained from infectious sap. As we now know, some  $10^5$  particles of virus are needed to cause a single infection, so that the quantity of virus obtained from a kilogram of plant tissue may be measured in grams instead of fractions of a milligram, as one might have anticipated. The reason for this singular lack of infectivity of plant virus particles is not completely understood even now. It may be, as some hold, that only a very small proportion of each preparation is potentially infectious—a view which, incidentally, casts doubt on the validity of much of the recent exciting work which has been carried out on plant viruses. It may equally well be due to a statistical effect reflecting the improbability of any individual virus particle reaching a suitable site in a viable plant cell, and then being able to initiate an infection. It may well be a combination of both of these, but at the moment it is not possible to tell with certainty. The most probable thing is that the chance of infection is small, but that not all particles are potentially infectious. However, it should be borne in



mind that all, or nearly all, workers in the field make the tacit assumption that all, or nearly all, of their virus particles are viable. This assumption may be unjustified.

From a study of the properties of crude virus in sap it was realized at an early stage that the tobacco mosaic virus was probably protein in nature; in 1933, Takahashi and Rawlins, by an inspired experiment, deduced that the virus was rod-shaped. The very fact that they were able to do this, using a simple technique involving the observation of the sap flowing through capillary tubes in polarized light, should, of course, have indicated that the quantity of virus material in plant sap was fairly large. About the same time, Bechhold and Schlesinger (1933) made an estimate of the size of the virus particles. They did this by a centrifugal method, which depended upon the estimation of virus activity, so that their results could be attributed directly to the virus in the plant sap, rather than to material, nonviral in nature, but associated with virus infection. Unfortunately, they assumed that the virus particles were spherical, but their experiments showed that the virus could be sedimented in ordinary laboratory high-speed centrifuges.

Shortly after this, Stanley (1935, 1936), who had been making an intensive study of the properties of the virus in impure sap and of the effects of various treatments on its infectivity, came to the conclusion that the infective agent was probably protein in nature, thus resembling the enzymes, and so he applied the purification methods which had been developed for the isolation of the latter. Using ammonium sulfate and acid as precipitants, he was able to isolate a protein having the characteristics of a globulin and having high infectivity. The material also had a crystalline appearance. Repeated recrystallization had no effect on the general properties of this material, so Stanley concluded that his preparations were essentially pure and consisted of the virus itself, even though the amounts which he isolated were so much greater than had been anticipated by other workers.

As is frequently the case in scientific investigations of this type, several others were working in the field at the same time. Notable among these was Best (1936) in Australia, who noted that a globulin-like protein, which had virus activity, was precipitated when the sap from infected plants was acidified. Also about this time Bawden and Pirie (1936), who were interested in the antigens present in virus-diseased plants, made partial purifications of potato virus X (potato latent mosaic virus) and found that their preparations consisted largely of protein.

The next advance made was that it was found that the tobacco mosaic virus was a nucleoprotein. Largely owing to the adoption of unsuitable methods for the drying of the virus preparations for analysis, Stanley had overlooked the presence of some 0.5 % of phosphorus on a dry weight basis in his preparations. This phosphorus, which is present as ribonucleic acid,



was discovered by Bawden and Pirie (1937a), and is now known to be very important for virus activity. Bawden and Pirie used methods similar to those used by Stanley, but employed other refinements, including the use of enzymes for the removal of traces of plant antigens from the virus.

Shortly after this, methods were developed for the isolation of the virus by means of the newly developed high-speed, air-driven ultracentrifuges (Bauer and Pickels, 1936), and these methods produced virus which was much more uniform and appreciably more infectious than was that made by salting-out techniques (Wyckoff *et al.*, 1937). It is now known that plants infected with tobacco mosaic virus contain more than one substance, and that salting out tends to produce artifacts which have served to cause the confusion which has existed and to some extent still exists in the field.

### *B. The Virus in the Plant*

The tobacco mosaic virus is present in diseased tobacco plants (Fig. 5) to the extent of several milligrams per gram of leaf tissue, the actual quantity being dependent upon the age of the plants, strain of virus, and general cultural conditions. In other hosts the yield is generally less, and may on occasion be much less. In tobacco plants a substantial portion of the virus is present as hexagonal plates (Fig. 1) which have been studied by a number of workers (Bawden and Sheffield, 1939) and have been shown by Wilkins *et al.* (1950) to consist very largely, if not entirely, of uniform length rods, some 300  $\mu$  in length, packed in hexagonal arrays. The structure of these crystals has been investigated very thoroughly by these workers (Fig. 6), and their results have done a lot to confirm the views put forward largely on electron microscopic evidence (Oster and Stanley, 1946; Oster *et al.*, 1947; Knight and Oster, 1947; Williams and Steere, 1951; Steere, 1957) that the virus consists of uniform length particles.

Needle-like inclusions, resembling Stanley's crystals, which are in fact *paracrystals* and only possess two-dimensional symmetry, are also to be found, mainly in the terminal cells of the leaf hairs. The tissues also contain a noninfectious protein (or proteins) related chemically and immunologically to the virus, but which does not contain nucleic acid. The ability of the latter material to disaggregate and reaggregate, and in doing so to engulf non-specific nucleic acid, has been the cause of much confusion. Certainly the early preparations of the virus must have contained much of this material, which may be a precursor of the virus.

Not all the virus material in plants is released when the plants are ground up. Extra material related to the virus is obtained from the solid residues if they are treated with cellulase or subjected to severe mechanical damage (Bawden and Pirie, 1945b).

FIG. 5. Tobacco mosaic virus on a young tobacco plant.

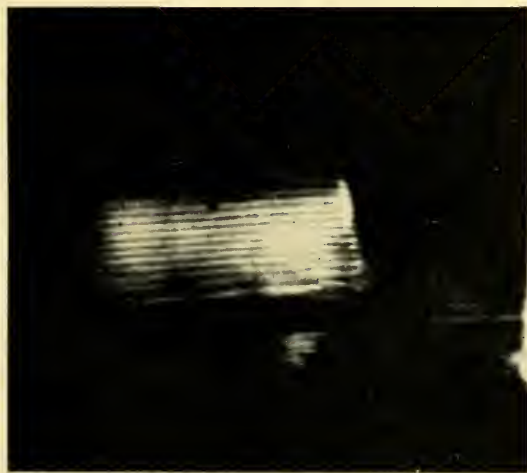
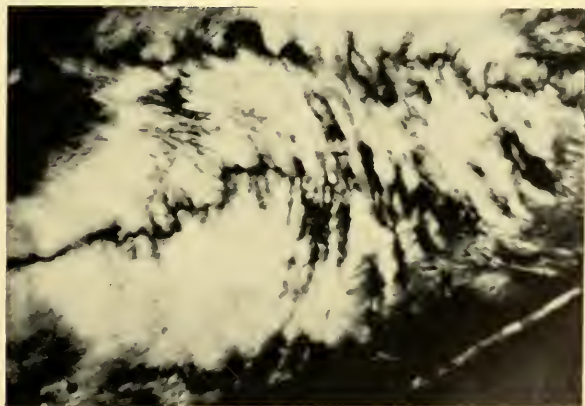


FIG. 6. Crystal of tobacco mosaic virus, photographed at right angles to the hexagonal plane in polarized light, and showing the alternating dark and light bands. The distance between dark bands is twice the length of the virus rods (Magnification:  $\times 2,100$ ). Photograph by Dr. M. F. Wilkins.

FIG. 7. Liquid crystalline ("bottom layer") tobacco mosaic virus oriented by flowing on a glass slide. Photographed in polarized light.





*C. Purified Preparations*

When the plants are ground up and the sap expressed, the uniform rods of virus may be isolated by several cycles of high-speed centrifugation alternated with low-speed spins. Such a preparation is characteristic of modern tobacco mosaic virus as it is usually investigated. If this type of preparation is allowed to stand at room temperature for any time as an aqueous solution, it almost invariably becomes contaminated with bacteria, whereas a similar preparation made by salt precipitation, although obviously aggregated, usually remains sterile. Apparently the rods, although uniform in appearance, retain a superficial layer of contaminating plant material. This layer is exceedingly difficult to remove, and contains, among other things, plant ribonuclease, nucleic acids, and host-specific antigens (Holden and Pirie, 1955; Pirie, 1956a). It should be remarked that such material constitutes but little of the total mass of the preparations, but its presence may account for some of the anomalous behavior of the virus suspensions. There is some indication that the contamination tends to be concentrated at the ends of the virus rods, and so effectively prevents end-to-end aggregation. Certainly treatments which tend to remove the contaminants usually result in such an aggregation, which is characteristic of the more drastically treated preparations. This aggregation, which is evidently an extension of the crystalline structure of the virus rods themselves (Bernal and Fankuchen, 1941a), is extremely difficult to reverse completely; a uniform suspension of rods cannot be obtained from such preparations, although, of course, greatly elongated rods are unstable even to small hydrodynamic forces. Larger forces will even disrupt the short virus rods (Oster, 1947).

Aggregation of the virus is the almost invariable consequence of the treatment of preparations with proteolytic enzymes or with salt or acid sufficient to precipitate the virus from solution.

Some substances, such as the strongly basic protein, pancreatic ribonuclease, combine extremely strongly with the virus, which is essentially acidic, and cause the formation of long fibres (Loring, 1942). When such an aggregation takes place, a secondary effect is also noted; that is the formation of *tactoids* (Bernal and Fankuchen, 1941a). These are cigar-shaped droplets of virus which have a higher concentration of protein than that in the bulk of the fluid, and these sink to the bottom of the container, giving rise to the "bottom layer," first noticed by Bawden and Pirie (1937a). These tactoids, which are equivalent to the "crystals" obtained by Stanley, are aggregates of rods having variable lengths, and so ordered that the long axes of the rods tend to be parallel to the long axis of the tactoid, although the shape of the tactoid precludes perfect parallelism throughout. In a plane at right angles to their length the rods are arranged in a two-dimensional, hexagonal, close packing, which is maintained even when the particles are

several diameters apart. The proportion of the material in the form of tactoids depends upon a number of factors, the principal one being the electrostatic repulsion between the rods, so that if swamping amounts of ions are present, as in strong salt solutions, or if the surface charge, which is mainly due to carboxyl groups, is reduced by lowering the pH, the number and the internal density of the tactoids increase and the "crystals" separate out.

The bottom layer is readily oriented by shear forces (Fig. 7) and it may be aligned by sucking into tubes and by gradual drying; these methods have been used for the preparation of much of the material used in X-ray investigations. It must not be thought, however, that bottom layer has unique properties. The pellets of unaggregated virus obtained by ultracentrifugation have very similar optical and physical properties, but because the material is composed of much shorter rods, these are not manifested so readily until the solutions are much more highly concentrated.

The unaggregated virus consists of rods which appear to be of relatively uniform length when examined carefully by a number of methods. It is true that there is considerable objection in some quarters to the idea of the complete uniformity of the length of the virus particles, but the evidence for such uniformity is more or less overwhelming, or at the worst much more convincing than the opposing view, which originated through the study of aggregated preparations. The exact length of the particles is, however, less certain, but it is of the order of 3000 Å. Measurements of particle lengths of this order of size present considerable practical difficulties. The most direct, and in many ways the most convincing one, is by electron microscopic observation. This has been brought to a high degree of refinement by Williams and his associates by the use of spraying and internal standards (Williams *et al.*, 1951). Spraying is, of course, by no means without its dangers, because the shear forces encountered may even break the virus rods, and the calibration of the internal standards also presents some problems. In addition, astigmatism of the electron optics involves systematic errors which must be corrected for. However, the results obtained by different workers have been in very remarkable agreement.

Another method for the direct measurement of the particle length is that employed by the King's College group (Wilkins *et al.*, 1950). These workers noted that the hexagonal plates found in plant cells were multiple twin crystals, having the virus rods arranged in a zigzag order along a line normal to the hexagon face of the hexagonal plates. The consequence of this type of arrangement was that, instead of exhibiting extinction in polarized light in a plane at right angles to the hexagonal face, the crystals exhibited two extinction positions at slight angles to this plane. Therefore, when viewed in one extinction position the edges of the crystals appear to



be banded. The light or dark bands, which alternate, can then be measured directly, and the particle length deduced, correcting for the angle between the extinction positions. The length observed was in fact about the same as that deduced from electron microscopy (3000 Å). (Fig. 6.)

Other methods used have been ultracentrifuge sedimentation combined with measurements of viscosity and diffusion, light scattering, and flow birefringence. All of these methods, which are discussed elsewhere, are in reasonable agreement, but cannot be regarded as being particularly precise.

A fairly recent method, which would seem to combine precision of measurement with some uncertainty of interpretation, is the measurement of the rotary diffusion of the molecules. This can be measured relatively accurately by following the relaxation of the molecules to a random order after they have been oriented by external forces. In this work the orientation was produced by an electric field, in which the molecules orientate themselves to produce as small a capacity as possible. On removal of the electric field, the particles relax to a random orientation, and this can be followed by observing the birefringence of the solution. In this way a rotary diffusion constant of  $333 \text{ sec.}^{-1}$  was obtained for zero virus concentration (O'Konski and Haltner, 1957). Making a number of assumptions as to the cross section and the hydration of the particles, a length of  $3416 \pm 50 \text{ Å}$  was deduced. Errors involved in the assumptions would tend to reduce this estimate and, of course, the values found are for the virus in solution. The electron microscope figures are based on observations of virus which has been drastically dried, a procedure which almost certainly results in shrinkage, and, of course, the X-ray estimates of diameter have been obtained on virus which has been more or less severely damaged by the intense radiation. These factors are often overlooked in assessing the relative merits of techniques of this type.

For the remainder of this chapter we shall assume a length of *about* 3000 Å or 300  $m\mu$  for the dry virus rods.

The diameter of the virus was for a long time thought to be 150 Å. This value was obtained from the X-ray diffraction data obtained by Bernal and Fankuchen (1941a) who found that the closest packing distance between the hexagonally arranged rods had this size. Since this time, X-ray techniques have advanced greatly, and instead of measuring the distances between the centres of adjacent rods, it is now possible to plot the distribution of density from the rod centers towards the periphery. By this means it is possible to show that the particles extend radially as far as 90 Å from the axis; that is to say, the particle diameter is 180 Å at its outermost edges, or 30 Å more than the distance between particle centers. This is in reasonable agreement with the once widely held view that the particles were hexagonal in cross section (if the particles were hexagonal in cross section with centers 150 Å apart, the maximum width of the particles would be 173 Å), but

Watson (1954) and later Franklin (1955a) have shown that the rods are helical, having 49 subunits arranged helically in every three turns of a helix of pitch 23 Å. The rod itself is hollow, with a hole of 20 Å radius extending throughout (Caspar, 1956b; Franklin and Klug, 1956; Huxley, 1957).

The present picture therefore necessitates the assumption of the existence of a groove, some 60 Å deep, between the helically arranged subunits, so that the particles fit into each other when close packed. The surface of the rod is probably studded with protuberances, rather like the appearance of a cob of sweet corn having pointed grains (Franklin and Klug, 1956). In spite of the fact that one might expect to observe this type of feature by electron microscopy, so far no surface detail of a convincing type has been seen.

The approximate molecular weight\* of the virus can be obtained by a number of methods, none of which are very accurate; it lies between  $45 \times 10^6$  and  $50 \times 10^6$ . For convenience we shall assume the latter figure, which is close to that obtained by a direct particle count by Williams and associates (1951).

#### *D. Optical Properties*

The tobacco mosaic virus, being a large nucleoprotein, has a high light absorption which is a summation of three major effects. First, owing to its very size, the virus absorbs light nonspecifically and its solutions are opalescent. This effect is most obvious in the ultraviolet region, as it increases markedly with decreasing wavelength. Owing to the presence of aromatic amino acids in the protein component, the virus has a specific absorption in the region around 280  $m\mu$ , and because of the nucleic acid it has a specific and high absorption in the 260  $m\mu$  region (Fig. 8).

These three separate effects combine to give a characteristic absorption spectrum which will vary somewhat with the apparatus used for the measurement (the effect of the scattered light will depend upon the acceptance angle of the measuring device used). The effect of the diffuse scatter can be eliminated more or less completely by orientating the virus, which can be done by shearing a suspension, preferably liquid crystalline, between silica plates (Perutz *et al.*, 1950). The orientated material then has an absorption spectrum characteristic of a nucleoprotein. This type of specimen has a marked dichroism, which is an indication of the nonrandom orientation of the constituent absorbing compounds in the virus.

Attempts have been made to reconstitute the absorption spectrum of the virus from the sum of the absorptions of the constituents (Reddi, 1957a).

\* The term "molecular weight" is used here and elsewhere to denote the weight in grams of  $n = 6.02 \times 10^{23}$  particles. Its use is not intended to imply that the particles are themselves molecular in the sense that, for example, glucose is.

This involves considerable difficulty because, among other things, it is known that the absorption spectrum of substances such as nucleic acids are not only dependent upon their compositions but are also dependent in some way upon the geometry of the absorbing systems.

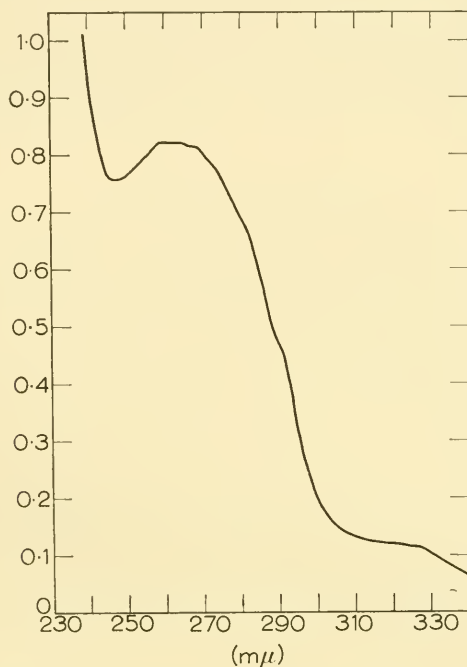


FIG. 8. Absorption spectrum of tobacco mosaic virus (0.27 mg./ml.) in a 1-cm. layer, recorded on a Cary No. 14 spectrophotometer. Note the shallowness of the trough at 245 mμ, and the high absorption at 310 mμ. Compare with Fig. 13.

The solutions of the virus are, of course, strongly birefringent when oriented by flow (Bawden and Pirie, 1937a) and, when sufficiently concentrated, they are birefringent spontaneously. This effect can be caused in suspensions of rods by pure form birefringence or, if the rods themselves are anisotropic, this can contribute toward the birefringence also. An attempt has been made to determine to what extent intrinsic birefringence is present in the particles by measuring the intensity of birefringence as a function of the refractive index of the suspending medium (Laufer, 1938). Very little intrinsic birefringence was found in the particles, but the main interest of this work is that the virus apparently tolerated a high concentration of anilin (Laufer and Robinson, 1949).

The virus, in fact, in the dry state, as "dry gel," has a weak positive birefringence, while the virus protein has weakly negative birefringence. The nucleic acid must therefore contribute to the birefringence in a positive sense, probably because the purine and pyrimidine rings are oriented parallel to the axis of the rods. This is in keeping with the ultraviolet dichroism of the particles (Franklin, 1955b).

The infrared dichroism of the virus has also been investigated and indicates that the polypeptide chains are in the  $\alpha$ -configuration, and run at right angles to the long axis of the virus particles (Fraser, 1952; Beer, 1958). It will be noted that this does not define the direction of the chains absolutely.

## VI. PROPERTIES OF THE VIRUS PROTEIN

It is usually assumed that the suspensions of the tobacco mosaic virus are uniform both in nature and composition. A very superficial survey of the published work on this virus will indicate that this is not altogether so. For example, it is quite easy to show that any tobacco mosaic virus preparation contains an appreciable number of mutants. It is quite simple, by taking single lesion isolates from *Nicotiana glutinosa*, to demonstrate one or more "yellow" variants per hundred isolates, and yellow variants are not the only ones present. On a more chemical level, simple treatment of the virus suspension at 0°C. at a pH of 10 results in the disintegration of 70 % of the particles, but leaves 30 % of the particles intact apparently indefinitely (Schramm *et al.*, 1955b; Harrington and Schachman, 1956). Moreover, the progeny of such alkali-resistant particles has a similar proportion of labile and resistant particles, the exact proportions possibly varying with the "strain" of virus examined.

On an even more sophisticated level, the amino acid analyses made on purified viruses tend to show deviations from the simple proportions anticipated, which are greater than one might expect on the basis of a model virus having uniform particles with a regular simple subunit. And, as has been mentioned, small amounts of plant contaminants are invariably present. It is, therefore, not surprising that some of the more critical workers regard the detailed analysis of viruses as probably misleading. The alternative is to hope that the structure may be uniform, at least to a major extent, but to bear in mind that there may be pitfalls. This is what we shall do, so that it is necessary to bear in mind that the following discussion refers to an idealized situation. This is, of course, the only way in which one can tackle this type of problem at the moment, and it is pertinent to remark here that much of the methodology in current use is suited to this type of approach. For example, both ultracentrifugation and X-ray diffraction analysis are techniques which automatically sample the greater part of any material



investigated. Simple analysis of elementary composition, and even of individual amino acids, also tends to do this when the precision of the method is not sufficient to distinguish between chance errors and real deviations from the results anticipated on the basis of a simplified structure. It may well be that the adoption of more refined and sensitive techniques may cause some modification in our ideas about the structure of the virus protein. The present data, however, give a reasonably satisfying picture of the over-all appearance of the virus, but possibly as seen through a slightly imperfect window.

### *A. Chemical Composition*

The virus consists almost entirely of protein and ribonucleic acid in the proportion 94.4 : 5.6 (the value 94 : 6 usually quoted is almost certainly incorrect; this will be discussed later). The general elementary composition is approximately: C, 50 %; H, 7 %; N, 16.7 %; S, 0.2 %; and P, 0.54 %. Variation in these values is reflected in the minor amino acid differences found in various strains. The amino acid and nucleic acid compositions are dealt with later.

### *B. Evidence Relative to the Existence of Substructure*

Although the virus behaves superficially as if it were composed of nucleoprotein rods having a molecular weight of  $50 \times 10^6$ , there is a lot of evidence that it is composed of uniform subunits of a less complex nature. Now it should be repeated here that there is considerable evidence, already mentioned, that the virus suspensions are *not* homogeneous. This does not, of course, necessarily imply that the various rods are made from different subunits; in fact, it is now accepted as a working hypothesis that the subunits composing the virus protein are all similar, if not identical. That this may not be altogether true may emerge from the subsequent discussion.

The idea that the virus was made up from small protein subunits first originated from the X-ray diffraction data of Bernal and Fankuchen (1941a). This concept has considerable appeal, because the idea of a unique protein having a molecular weight in the tens of millions seems improbable, to say the least. Evidence for the existence of a fairly small subunit (or set of subunits) has come from several observations.

The virus rods are acidic, moving to the anode on electrophoresis at all pH's above about 3.5, presumably because they have carboxyl groups on or near the surface. Oster and Grimsson (1949) measured the uptake of basic dyes by the intact virus and found that, for example, some 6500 molecules of the basic dye acriflavin were taken up by one virus particle, while no



acidic dyes were taken up unless the virus was denatured. Ginoza and Atkinson (1956) have made similar measurements using the basic dye safranin, of which about 3300 molecules are fixed per virus particle. They also noted that after treatment with carboxypeptidase, about twice as many safranin molecules were bound. the extra number by a site which has a distinctly different affinity for the dye than has the original site.

A number of measurements made by Miller and Stanley (1941) are also relevant to this matter. The number of free amino groups in the virus was estimated by deamination with nitrous acid, and by acetylation, and they came to one for every 9000 to 10,000 molecular weight. As an incidental observation arising from the controls to these experiments, these authors noted the presence of acetyl groups in the intact virus. The number which they estimated was about four per 19,000 molecular weight. So far only one of these four has been identified, namely, the *N*-acetyl serine termination of the peptide chains, which will be mentioned later.

The amino groups were also estimated by Fraenkel-Conrat (1953) by the use of  $C^{14}$ -labeled *N*-carboxyleucine anhydride. This reacts with amino groups, including the  $\epsilon$ -amino groups of lysine. About a thousand groups were introduced into the virus without changing its infectivity or electrophoretic mobility.

In 1944, Best and Lugg determined various constituents of highly purified tobacco mosaic virus; from their results one can deduce that there is one cysteine residue for 16,000 molecular weight, and two tryptophan residues per 20,000 molecular weight. These figures have stood the test of time, and are of considerable use in deducing the size of the minimum chemical subunit.

The sulfur in the type strain is in the form of cysteine entirely (Ross, 1940; Best and Lugg, 1944; Knight, 1947), and reacts with iodine quantitatively to give a stable iodine-containing compound having the grouping  $-S-I$  (Fraenkel-Conrat, 1955). Much has been made of the lack of reactivity of the  $-SH$  group, but this would appear to be due entirely to its isolation from its nearest neighbor by a distance of at least 20 Å.

The  $-SH$  grouping also reacts with compounds of the type  $CH_3HgCl$  to give substituents of the type  $-S-HgCH_3$ . Such groupings having a heavy mercury atom are of considerable use in interpreting the X-ray diffraction measurements (Franklin and Holmes, 1956). One mercury atom is bound per 18,000 molecular weight and at a radius of 69 Å. The results obtained with lead are less clear-cut. The virus will take up one lead atom from lead acetate per 17,000 molecular weight and still remains soluble, but the lead is found in similar amounts at two levels in subsequent X-ray analysis (Caspar, 1956b). One set of atoms is at a radius of 84 Å, which is at the periphery of the virus, while the other set is at 25 Å radius, which is 15 Å *inside* the position of the nucleic acid phosphorus atoms. The superficial lead

atoms are presumably chelated, and it is difficult to visualize how they are bound unless either there are two types of subunit, only one of which can combine with lead, or, more probably, that the true subunit is paired, and that the lead atom chelates groups from each of these true subunits. The "chemical" subunit would then be half of the "physical" subunit. A very convincing argument for the existence of such subunits was put forward at one time, but was later withdrawn without comment (Franklin, 1955a).

The idea of a true subunit of about 35,000 in molecular weight, and composed of two similar subunits alternating would also fit in very nicely with the data obtained with the closely related cucumber virus 4, which has one tryptophan residue in a subunit of this size (Knight, 1949), and which has a very similar X-ray diffraction pattern (Holmes and Franklin, 1958).

Perhaps the most convincing evidence that polypeptide chains of molecular weight 17,000–18,000 exist comes from the work of Harris and Knight (1955), who observed that carboxypeptidase liberates one, and only one, amino acid residue from a number of strains of tobacco mosaic virus, and that this amino acid is threonine. Carboxypeptidase only attacks the C-terminal end of polypeptide chains, and the action of the enzyme is stopped in this case after the first link is broken, because the second one happens, quite by chance, to be resistant, the terminal sequence being prolyl, alanyl, threonine, and prolyl links are not broken by the enzyme. The number of threonine molecules liberated indicates that there is one for every 17,000 molecular weight.

This quantity of C-terminal threonine has also been found by hydrazinolysis, although rather large corrections have to be made in this case (Braunitzer, 1955). It has also been reported that the N-terminal amino acid was proline, and that one N-termination was to be found for every 17,000 molecular weight (Schramm *et al.*, 1956). As will be seen later, this is probably not a true end group, but may result from an unusually specific chemical hydrolysis, and so may also give an indication of the length of the polypeptide chains.

The bulk of the evidence, therefore, is that the tobacco mosaic virus has polypeptide chains of about 17 to 18 thousand in molecular weight, possibly in pairs, all of which have similar, if not identical, structures, and that they are arranged in some regular order to form a tubular molecule containing a core of nucleic acid, and composed of about 2800 of such subunits.

### *C. The Amino Acid Composition of the Protein of a Typical Tobacco Mosaic Virus*

Although the tobacco mosaic virus has been investigated more intensively than any other virus, quantitative data on the amino acid composition are

surprisingly scarce. In part this is due to the fact that accurate and reproducible methods for the analysis of amino acids have been developed comparatively recently, while much work was done before this by the tedious, and relatively inaccurate, methods available before amino acid chromatography had reached its present state of development. The best of the earlier methods for the general analysis of amino acids was undoubtedly the microbiological assay technique, which has been used extensively by Knight, and which has produced much interesting comparative data between virus strains. It is, however, a method which assays the amino acid in a hydrolyzate, using the former as a growth factor in an otherwise complete medium for the organism used for the assay, and is thus inevitably suspect, because the hydrolyzate may contain inhibitory substances or even growth-promoting substances. There are, in fact, indications that the method may overestimate some amino acids and underestimate others in as complex a substance as a virus. As it happens, analyses of tobacco mosaic virus, and indeed of the same strain as used by Knight (1949), have been made for an entirely different reason, and published by Newmark and Fraser (1956), and, using these and Knight's data, it is possible to obtain a fairly reasonable and complete idea of the actual amino acid composition of the virus. The analyses made by the later workers were obtained by the Moore and Stein ion-exchange chromatographic technique, which has been investigated most thoroughly and is generally accepted as being reliable. There are, however, one or two gaps in these data which are filled in from the microbiological assay data, most of which are in good agreement with the ion-exchange results, but some of which differ very appreciably from the latter. However, in this way one can obtain a reasonably good idea of the amino acid composition as a whole.

#### *D. The Polypeptide Subunit*

Making the assumption that all the polypeptide chains are the same, and that their length can be estimated from the information given earlier, one may fit the amino acid analyses so that the chain contains integral numbers of residues. If one does this, one finds that the chains have a molecular weight of about 17,000 (the exact figure cannot be calculated directly from the data because of the existence of amide groups in the molecule, and possibly of cross linkages which involve elimination of water). These chains contain about 145 amino acid residues.

The chains contain 1 residue of cysteine. This is evidently on or near the surface of the virus because it is reactive to substances such as methyl mercuric chloride and iodine. The cysteine residue, presumably because of its isolation, is not oxidized readily, and may be substituted by an iodine atom to give an —S—I substituent. This type of grouping is usually very

reactive, and gives rise to —S—S— linkages, but in the tobacco mosaic virus is prevented from doing this until denaturation or disaggregation has taken place.

The type virus contains 2 tryptophan residues per subunit, although, as mentioned above, the related cucumber viruses 3 and 4 have only half a residue per 17,000 molecular weight. There are also 2 lysine residues. One of these is blocked so as to resist the action of fluorodinitrobenzene, even after the virus has been disaggregated (Fraenkel-Conrat and Singer, 1954). Presumably this may mean that the  $\epsilon$ -amino group is already substituted by one of the acetyl groups detected by Miller and Stanley (1941). This observation would also explain the fact that only one lysine peptide is liberated by trypsin instead of the anticipated two (Schramm and Anderer, 1955). The other lysine  $\epsilon$ -amino group is presumably available and is one of the two groups substituted by carbobenzoxy, phenylureido, *p*-chlorobenzoyl, and benzylsulfonyl groups, a substitution which does not involve appreciable loss of virus activity (Miller and Stanley, 1942). This substitution does block the two free amino groups which react with nitrous acid, and it increases the electrophoretic mobility by about 10 % at neutral pH values. It is surprising that these basic groups do not react readily with acidic dyes, but this may be due to their steric arrangement. It is also a matter of some interest to know what the second amino group is attached to, because only one would be anticipated, the terminal amino group of the polypeptide chain being blocked by an acetyl group. It is, of course, always possible that the lack of reactivity of the second lysine  $\epsilon$ -amino group is due to steric effects, but this would hardly explain the results of the tryptic digestion.

There are 4 tyrosine residues in the subunit. Proline accounts for 6 residues, thus allowing some 6 major bends in the polypeptide chains. One of these residues is near the C-terminal end of the chains, while another is exposed very readily by the action of 5 % trichloroacetic acid at 85°C. (Schramm and Braunitzer, 1953).

There are some 6 glycine residues, and 7 each of valine, phenylalanine, and isoleucine. Next come 9 arginine residues, which are probably the most important factors in estimating the actual chain size, because each gives rise on tryptic digestion to a break in the chain, which can be detected by the isolation of a peptide containing 1 arginine residue. Nine peptides of this type are in fact found (Fraenkel-Conrat and Narita, 1957). (This is, of course, easily demonstrated by a colorimetric spray for arginine). The proportion of arginine is rather low for a nucleoprotein, but is more than adequate to neutralize the 3 phosphate groups corresponding to each subunit. The remaining 6 guanidino groups of the arginines must be well buried in the protein, because the virus as a whole has a predominantly acidic character. This also



accounts, no doubt, for the resistance of the virus to tryptic digestion when in its native form.

Leucine accounts for 11 residues; serine, threonine, and glutamic acid each for 14; alanine for 15; and finally aspartic acid has 17 residues.

It will be noted that there is no histidine, and that as there are 9 arginine residues and 1 free lysine  $\epsilon$ -amino group, trypsin is likely to produce only 11 peptides, when acting on the denatured or disaggregated protein. Nine of these are likely to end with arginine, 1 with lysine and 1 with threonine. This is essentially what has been found experimentally by the Berkeley group, though they anticipated a larger number of peptides (11 arginine, 2 lysine).

This number of peptides has also been found by Schramm and Anderer (1955) who also only found 1 lysine peptide instead of the expected 2.

The number of carboxyl groups is very large. There is 1 terminal carboxyl group of threonine, and aspartic and glutamic acids provide a further 31. Of these about 19 are amidated (Best and Lugg, 1944; Black and Knight, 1953), leaving some 13 carboxyl groups to account for the electrophoretic charge. Oddly enough only 1 of these can bind safranin and 2 can bind acriflavin. After carboxypeptidase action 2 safranin molecules are bound, but the two sites are by no means equivalent. This might suggest that the groupings involved are dissimilar, but a more probable explanation is that one site is less readily accessible. Certainly there is not much likelihood that the acidic groups of the nucleic acid are involved in this reaction.

On the action of dodecyl sulfate 2 more acidic groups are liberated (Harrington, Levy, and Schachman, quoted by Fraenkel-Conrat, 1957). These are not due to the nucleic acid, and presumably represent carboxyl groups, which may have been hydrogen-bonded, possibly to some of the enormous number of hydroxyl groups (28 alcoholic, 4 phenolic) present in the virus.

The carboxylic groups of the virus seem to be the main factors which solubilize it. They are responsible for the interparticle repulsion which is swamped by quite low (0.5 *M*) ammonium sulfate concentrations, and when the groups are caused to become undissociated, at pH's about 3, the virus completely loses its solubility in water. The inference from this is that the surface of the virus has relatively few hydrophylic groupings, apart from the carboxyl groups and the 2 amino groups, which account for only 15 of the 145 odd amino acid residues present in each subunit.

Since the foregoing was written a detailed and careful investigation of the composition of the tobacco mosaic virus protein has been published by Ramachandran (1958). The figures which he has found differ slightly from those published by Newmark and Fraser, who used a method which was essentially the same. The results differ primarily in the quantities of glycine,



proline, and alanine, of which he finds relatively less, and of isoleucine, phenylalanine, leucine, serine, arginine, threonine, and glutamic acid, of which he finds somewhat more. He has expressed his results in the form of integral numbers of residues per subunit, and deduces the existence of a subunit having a molecular weight of 18,420 having 164 residues, these being: 19 (16) aspartic acids; 17 (15) threonines; 18 (16) serines; 8 (7) prolines; 17 (14) glutamic acids; 6 (5) glycines; 15 (13) alanines; 14(12) valines; 9 (8) isoleucines; 13 (11) leucines; 4 (3) tyrosines; 8 (7) phenylalanines; 2 (2) lysines; 11 (9) arginines; 2 (2) tryptophans; and 1 (1) cysteine; plus 20 amide groups. The figures in parentheses are the result of altering the data of Ramachandran in such a way as to make arginine have the more probable value of 9 residues, and then fitting the rest of the data to the nearest whole numbers of residues. It will be noted that the size of the subunit is then about the same as that deduced from the data of Newmark and Fraser (141 amino acid residues compared with 145), but that the composition is slightly different.

It should be remarked here that if expressed on a weight basis, the quantity of arginine found by Ramachandran (1958), Newmark and Fraser (1956), and also by Black and Knight (1953) is essentially the same, so that if the data obtained by tryptic digestion (in the same laboratory, and confirmed elsewhere) are to be believed, there must be a systematic deviation in the results, which makes the subunit appear to be slightly too large. Alternatively, of course, there may be some structure of an unknown type which may prevent 2 of the arginine peptides from being released by trypsin digestion. This could be confirmed by making semiquantitative analyses on the 9 arginine-containing peptides. The virtue of counting the actual numbers of residues in each subunit, rather than attempting to deduce it from quantitative estimates, should be evident, and any estimate of the size of the protein subunit based upon such evidence should be very reliable.

With regard to the possible snags in such a procedure, it has been noted above that one of the two lysine groups seems to be blocked, both with regard to trypsin action, and also to chemical substitution of the  $\epsilon$ -amino group, and a reason for this blocking has been suggested.

A number of peptides have also been obtained by the action of pepsin and of chymotrypsin, and they are being investigated. One such peptide has been obtained by the action of both of these enzymes separately, and it is thought to represent the blocked N-terminal sequence of the protein, which has been referred to earlier (Narita, 1958b).

### *E. The Chain Ends*

The carboxylic end of the polypeptide chain has already been mentioned, and is -prolyl-alanyl-threonine. A hexapeptide containing this structure as

the C-terminal end was isolated from enzymatic (chymotrypsin) digests of several strains of tobacco mosaic virus, and, in addition, a similar pentapeptide containing prolyl-alanine as its C-terminal end was isolated from virus which had been treated previously with carboxypeptidase. Thus there is every reason to suppose that this hexapeptide does in fact represent the C-terminal sequence of the virus. The sequence of this peptide has been determined by finding the N-terminal amino acid, which is threonine, and then finding the relative positions of two of the remaining amino acids by partial acid hydrolysis. The whole sequence is threonyl, seryl, glycyl, prolyl, alanyl, threonine (Niu and Fraenkel-Conrat, 1955a,b). The Holmes ribgrass virus gives a similar peptide which has not yet been fully analyzed. Its sequence is threonyl (threonyl, alanyl), prolyl, alanyl, threonine, the sequence in parentheses being uncertain.

The determination of the nature of the other end of the chain gave much more trouble. As Miller and Stanley (1941) found, the only groups reacting with ketene and similar reagents were the basic groupings on the surface of the virus, one of which is certainly an  $\epsilon$ -amino group of lysine. Treatment of the virus with reagents such as fluorodinitrobenzene had no success in revealing any  $\alpha$ -amino groups in the virus, and there was a considerable tendency to regard the  $\alpha$ -amino group as blocked by being linked in some way to the rest of the chain so that it was not available. A possible way in which this could occur is by the formation of an amide link to a carboxyl group, of which there are plenty.

For some time it was thought, because of experiments reported by the Tübingen group, that this end was occupied by proline. In order to reveal it the latter had to subject the protein to rather harsh treatment. Using hot trichloroacetic acid for preliminary hydrolysis, they detected some 2300 proline residues as the dinitrophenyl derivative, and also as the thiohydantoin, in which case some 2400 residues were estimated, taking into account the considerable losses in this estimation. As it is now fairly certain that the N-terminal end is acetylated and consists of *N*-acetyl serine, it might appear that the German workers stumbled across an unusually specific chemical hydrolytic method in this work (Schramm *et al.*, 1955c).

The problem of the missing N-terminal end of tobacco mosaic virus now seems to be fairly well resolved. The Berkeley workers originally thought that this end was probably linked back on to the main chain, forming a ring at one end, and attempts were made to isolate the piece of chain containing this cyclic structure. Enzymatic digestion was used to break down the chain, and as it was considered useful to try to isolate the fragment by virtue of its having one carboxyl group at one end, and no terminal amino group, chymotrypsin and pepsin were used for the digestion, in the hope of producing a neutral peptide, rather than trypsin, which would

leave lysine or arginine at the carboxyl end. This is a trick calculated to facilitate the separation, which was carried out on a sulfonic acid (Dowex 50) ion exchange resin. Peptides containing no basic group are not retained by such a resin at all strongly, and in this way Narita (1958b) was able to isolate adequate amounts of two peptides, one from each enzymatic digest. These peptides, had no N-terminal amino acid residues, but were both found to be acylated. The acyl group was isolated as the hydrazide and found to be an acetyl group. The peptide isolated from each digest contained both serine and tryptophan only, in approximately equivalent amounts, with tyrosine as the C-terminal end. There are no quantitative data which would show that there is one such acetyl peptide per chain, and, as Narita has remarked, it is possible that this peptide may come from the blocked  $\epsilon$ -amino group of lysine. It does, however, seem quite likely that the peptide really represents the end of the peptide chain, which is probably *N*-acetyl, seryl, tyrosyl-. The possibility of the *N*-acetyl serine arising from *O*-acetyl serine by *N*→*O* migration seems to have been resolved satisfactorily by the isolation of the same peptide under both acid and neutral conditions, but this migration might take place at an early stage in the development of the virus. Whichever position was originally occupied by the acetyl group, however, it is certain to cause complications in the theory of the "coding" mechanism involved in protein synthesis. Neither acetic acid nor *N*-acetyl serine is an amino acid, while *O*-acetyl serine would be a new amino acid.

It is interesting to recall here that the presence of acetyl groups was discovered in tobacco mosaic virus 17 years before the acetyl serine residue was recognized.

### *F. The Position of the Nucleic Acid*

It is now clear that the main function of the protein component of small viruses is to protect the nucleic acid part. It became evident some time ago that several viruses had their nucleic acid located below the surface (Markham, 1951; Hershey and Chase, 1952; Watson, 1954). Since this time several workers have shown that the nucleic acid of the tobacco mosaic virus occupies an axial position.

The position of the nucleic acid may be revealed by a number of methods. Stahmann and Kaesberg (1955) were the first persons to detect it as an axial thread in virus which had been freeze-dried (Rice *et al.*, 1953), and shortly after this Hart (1955a,b) found that it could be demonstrated by the action of the anionic detergent, dodecyl sulfate, on the virus in solution. Dodecyl sulfate had been found by Sreenivasaya and Pirie (1938) to split off the protein of the virus, and it has since been used as a convenient method for the preparation of the virus nucleic acid. Dodecyl sulfate probably competes with

the virus nucleic acid for the basic groups of the virus, and so allows the protein to become free. Certainly one of the most intriguing things which it does do is to start from *one* end of the virus rods, thus showing dramatically that the rods have two different ends. As the attack proceeds the virus is peeled off and the nucleic acid is allowed to protrude. This can be observed by electron microscopy (Hart, 1955b), and the composition of the thread can be determined by acting on it with enzymes. It is, in fact, unstable to ribonuclease. Although a substantial proportion of the protein coat may be removed, the virus retains its infectivity unless the nucleic acid is destroyed by some means or other.

Schramm *et al.* (1955a, b) used another of Pirie's techniques to degrade tobacco mosaic virus. Pirie noted that the virus was very unstable to dilute alkali (Bawden and Pirie, 1937a, 1940) and Schramm *et al.* used carefully adjusted alkaline solutions to degrade the virus. The pH required is about 10.3 and the protein is stripped off either at 0 or 35°C. Presumably because the  $\text{—OH}^-$  ions are so small, the attack proceeds all over the surface of the virus, and small native protein fragments are released. There is some indication, however, that certain regions on the virus rods are more susceptible to the action of alkali, but the dissolution is complicated by the tendency of the small protein particles to reaggregate (Harrington and Schachman, 1956). In this case, too, the nucleic acid is revealed as an axial thread, and while the latter is intact, the rods, eaten away as they are, still retain their infectivity. The alkaline solutions also tend to break down the virus nucleic acid, but at pH 10.3 the action is very slow.

The position of the nucleic acid was determined still more accurately by Franklin (1956a), who, by X-ray diffraction, measured the radial density distribution of the virus rods and also of the nucleic acid-free polymerized A protein (which will be mentioned later). She found that the density due to the phosphorus atoms of the nucleic acid was located at about 40 Å from the axis of the rods. This distance is well within the part of the rod occupied by protein, which extends to within 19 Å of the axis, so that the nucleic acid is in fact buried in the protein. This is a very important observation, because it means that the two components must share certain structural regularities.

The nucleic acid contributes positively to the birefringence of the rods, so that the structure of the virus nucleic acid is very unlike that of deoxyribonucleic acid (Franklin, 1955b), which is strongly negatively birefringent.

### G. The A-Protein

Treatment with alkali is also used to produce native virus protein or "A"-protein for experimental work (Schramm *et al.*, 1955b). The A-protein is actually a mixture of substances in considerable flux. The smallest fragments



consist of particles having a sedimentation coefficient of about 4.6 *S* and a molecular weight of about 90,000, i.e., they represent about 1/500 of the protein of the virus, and as we shall see must be made up from some 6 (or 3) subunits. The A-protein is only really stable in a dispersed form in alkaline solution; on acidification to pH 6 it begins to aggregate and forms short cylinders having a hollow axis. Eventually it turns into fibers which resemble the virus in shape, but which have no definite length. They also have an X-ray diffraction pattern very much like the intact virus (Franklin, 1955b). The rods are, however, very unstable to heat compared with the whole virus (Schramm and Zillig, 1955), and dissolve very much more readily in alkaline solutions. In fact the polymer of the A-protein is only really stable over the pH range 2.5–6. Over this range, the mobility on electrophoresis is the same as that of the intact virus, presumably because the surface charge distributions are the same, even though the total number of charged groups in the molecules is different. This effect has been shown for at least three strains of the virus (Kramer and Wittmann, 1958).

Outside the zone of stability of the polymer the mobility changes, presumably because of the breakdown of the rods and the subsequent exposure of other charged groupings which had been hidden previously. This is of course only one of the instances where it has been shown that the nucleic acid of plant viruses does not appear to contribute to their electrophoretic mobility.

In the presence of nucleic acids and even of synthetic polynucleotides and partly degraded nucleic acid the A-protein may be polymerized in such a way that it actually includes the former (Hart and Smith, 1956), and gives rise to rods which resemble virus in their greater stability. In fact, if the polymerization is carried out in the presence of carefully prepared virus nucleic acid (Fraenkel-Conrat and Williams, 1955), not necessarily even from the same strain of virus (Fraenkel-Conrat and Singer, 1957), the so-called "reconstitution" ensues when a small proportion of material having infectivity is formed. When a mixed reconstitution is performed, the progeny is decided by the donor of the nucleic acid component. This phenomenon is discussed elsewhere.

### *H. The X-Protein*

Infected plants themselves contain a protein (or proteins), the X-protein of Takahashi and Ishii (1952a,b, 1953), Jeener and Lemoine (1953; Jeener *et al.*, 1954) which resembles the A-protein in many ways. This protein was first recognized by Bawden and Pirie (1945b, 1956), who thought it was a small form of the tobacco mosaic virus nucleoprotein. The methods which they used for the isolation of this protein were, however, such as to cause a certain amount of polymerization to take place, and so their material



invariably contained nucleic acid, probably of plant origin. Later workers have tended to eliminate this nucleic acid by their manipulations, and there is little doubt that the X-protein is normally devoid of this substance. Some workers have identified a number of proteins of this type by working at pH's at which the X-protein is in a state of flux (Commoner *et al.*, 1953), but there seems to be every reason to think that only one basic substance is involved, which is capable of aggregation and dissociation.

The precise nature of the X-protein is as yet uncertain (Delwiche *et al.*, 1955). It is quite evident though that it resembles the "top" component of the turnip yellow mosaic virus (q.v.) in many ways (van Rysselberge and Jeener, 1957).

Amino acid analyses which have been made on the X-protein by Newmark and Fraser (1956) indicate that this protein has the same amino acid composition as the protein isolated from the whole virus.

### *I. Effect of Heat on the Virus*

The tobacco mosaic virus has long been recognized as being unusually heat-stable. This reputation has been vastly over-emphasized, largely because the heat denaturation was originally followed by biological methods, and not only is the denaturation an exponential, or nearly exponential, process, but it is now known that the nucleic acid of the virus is itself infectious, and is moreover much less susceptible to the action of heat.

Actually the virus is denatured relatively readily by temperatures of 75°C. or thereabouts, although this is much less than the 36°C. required to denature the A- and X-proteins. In the presence of salt, the nucleic acid is then released from the protein and remains in solution, while the denatured protein clumps and settles from solution (Cohen and Stanley, 1942). The course of this denaturation has been followed by electron microscopy by Hart (1956). The denaturation process resembles the melting of the protein part of the rods from one end (this is additional evidence that the rods are not symmetrical in their length), and eventually all the protein is converted into spherical objects having about the same volume ( $53,000 \mu^3$ ) as the virus. The nucleic acid is ejected during this process, and then, presumably, the spheres of protein clump to give the coagulum of denatured protein.

Apart from the action of alkali and dodecyl sulfate, the virus is extremely stable to all manner of aqueous solutions including many proteolytic enzymes, although, of course, the action of carboxypeptidase is an exception. Solutions of the A-protein and of the denatured virus protein are, however, reasonably readily digested by enzymes, and it is in this way that much of the structure of the virus protein has been elucidated.

### *J. Reconstitution and the Infectivity of Virus Nucleic Acid*

It has been realized for a considerable time that the nucleic acid of viruses was probably the carrier of the "information" necessary for virus activity, and that it was wrapped in a protein shell in order to render it less liable to the action of extraneous influences. The nucleic acid of the virus may be obtained free, or nearly free, of protein by heating the latter in solution, or by acting on the virus with anionic detergents, or, as has been found quite recently, with phenol.

The nucleic acid so obtained is highly polymerized and seems to contain all the nucleotides present in one virus particle per nucleic acid particle. That is to say, the particles have a molecular weight of the order of 3 million. This has been determined by light scattering, while the other physical properties are in keeping with this value. If kept, the nucleic acid depolymerizes spontaneously, presumably because it is sufficiently contaminated with the very stable enzymes which are more or less ubiquitous, and which are invariably present in nucleic acid preparations.

Some years ago, Fraenkel-Conrat and Williams (1955) were able to demonstrate that the A-protein of Schramm, if allowed to polymerize in the presence of freshly prepared virus nucleic acid, was able to form aggregates—particles resembling virus in physical properties, and having reasonable stability, which were much more infectious than was either of the constituents separately. The order of infectivity was small, even compared with that of the original virus preparations, which are themselves very uninfected on a weight basis. This caused a certain amount of unfavorable comment and skepticism about the real meaning of the results, but by and large the phenomenon has become accepted because it is repeatable.

#### *1. Infective Nucleic Acid*

The nucleic acid of the tobacco mosaic virus was itself shown to be capable of initiating infection by Gierer and Schramm (1956a,b) and a similar, but less-detailed study was made simultaneously by Fraenkel-Conrat (1956). The idea that the nucleic acid might be the infectious agent was not a new one, but the general opinion was that it might be too unstable to exist in an infectious form for any time. The fact that the nucleic acid is unstable is easily seen from the failure of Fraenkel-Conrat and Williams (1955) to observe its infectivity in their reconstitution experiments, which were in fact controlled by testing the infectivity of the nucleic acid. However, Gierer and Schramm, using a new method for the isolation of the nucleic acid coupled with careful control of temperature, managed to make an infectious nucleic acid preparation.

The nucleic acid was isolated by shaking a 10 % solution of the virus in water at pH 7.3 with an equal volume of water-saturated phenol. Phenol

is an excellent solvent for proteins, but the nucleic acid remains in the aqueous phase. The extraction with phenol was repeated twice more, and excess phenol was removed from the water by means of ether. The aqueous solution was tested on *Nicotiana glutinosa*. The infectivity was then found to be equivalent to 0.1 % of that of an equal amount of nucleic acid as it existed in untreated virus. When one considers the relative lack of infectivity of untreated virus, and that some  $10^8$  particles of nucleic acid were therefore needed to cause a single infection, a certain amount of faith is required in the interpretation of the results, particularly if one considers that the absence of active virus can only be controlled by indirect methods.

In particular, the infectivity possessed by the nucleic acid preparations is very labile to the action of pancreatic ribonuclease at concentrations of the latter which have little effect on whole virus (Gierer, 1957). Conversely, the activity is much less affected by antiserum to the whole virus, though it is indeed surprising that the serum used did not contain enough ribonuclease to inactivate the nucleic acid.

A rather more striking piece of evidence in favor of the theory that the nucleic acid is itself infectious is given by Schramm and Engler (1958), who state that the course of infection with nucleic acid is different from that when whole virus is used. Using the ability of the virus to produce systemic infection in tobacco as a sensitive test for the production of virus, they found that they were able to detect virus in tobacco plants infected with the nucleic acid about 20 hours after inoculation. In plants inoculated with whole virus this state was not attained until some 12 hours later. This suggests that the nucleic acid, not having to emerge from its protein sheath, is able to multiply without so long a time lag.

Unfortunately, the figures which Schramm and Engler produce to substantiate this claim contain a number of improbable features. First, they claim that they get 54 % of infections with solutions which only contain one particle per millilitre. Apart from the inherent improbability of this figure, which is many orders of magnitude greater than normally obtained, they give figures showing that only 4 plants out of 25 inoculated with virus a million times as concentrated became infected. It seems likely that their experiments contained a source of uncontrolled error. Leaving this aside, however, there would seem to be no reason why one should query the fact that detectable virus appears some 12 hours earlier if nucleic acid is used for the inoculations rather than whole virus. A similar conclusion was reached by Siegel *et al.* (1957) on the basis of measuring the ultraviolet light sensitivity of tobacco mosaic virus and virus nucleic acid after inoculation into plants. The nucleic acid used by these workers was prepared by treatment of the virus with dodecyl sulfate, as was that made by Fraenkel-Conrat (1956), and in both cases the low infectivity observed by Gierer and Schramm was

confirmed. Fraenkel-Conrat observed that coprecipitation with virus protein increased the apparent infectivity several hundredfold. It would appear that this step of "reconstitution" would be a useful procedure when studying the biological activity of virus nucleic acids.

### *K. The Action of Various Agents upon the Infectivity of the Virus*

As has been mentioned, the present evidence is that the infectivity of the tobacco mosaic virus resides in its nucleic acid component. If this is so, then chemical or physical agents which inactivate the virus must act in one of two ways. Either they must modify the nucleic acid component so as to make it inactive, or they must interfere with the virus protein in such a way as to prevent the emergence of the nucleic acid, which must presumably precede the multiplication of the virus. Quite recently it has been shown that the nucleic acid from the tobacco mosaic virus, if inoculated into plants, causes the production of infectious virus after a time lag of 19 hours, while whole virus inoculated similarly takes at least 10 hours longer to start multiplying (Schramm and Engler, 1958). Thus any treatment which might interfere with the removal of the protein coat of the virus will cause inactivation in some degree. This could explain, for example, the effects of substituting various chemical groupings on the surface of the virus (Miller and Stanley, 1942) or of the indirect action of ionizing radiation on dilute solutions of virus (Lea *et al.*, 1944). The direct action of radiation of this type is probably due to direct hits on the nucleic acid core of the virus (Buzzell *et al.*, 1956) and a dramatic demonstration of this has been given by Lauffer and his colleagues, who showed that the action of X-rays on the whole virus could not be detected by viscosity measurements, but that the nucleic acid prepared from irradiated virus was appreciably disaggregated (Lauffer *et al.*, 1956).

Perhaps the most important single agent used in inactivation studies is formaldehyde. Formaldehyde, of course, is not a substance of much use for the inactivation of tobacco mosaic virus, but as it is a substance used for the inactivation of other viruses the chemistry of the action is of general interest. (We once had occasion to examine plant material originally imported into the United States of America in the form of sap, containing about 5 % v./v. of 40 % formaldehyde solution to render it "sterile." After being stored for some 2 years under these conditions viable virus was isolated without difficulty.)

The action of formaldehyde on proteins, and in particular nucleoproteins, is exceedingly complex. The various reactions which it causes have been reviewed at length by French and Edsall (1945). As far as the infectivity of tobacco mosaic virus is concerned the inactivation follows first-order



kinetics (Cartwright *et al.*, 1956). This probably means that one particular action is limiting, rather than that one formaldehyde molecule will inactivate one virus particle. One of the striking observations about the action of formaldehyde was that it was, in part, reversible. Formaldehyde does in fact react reversibly with amino groups or, to be more precise, it reacts with unionized amino groups to give a compound of the type  $R-NH-CH_2OH$ . At pH values below that necessary for the ionization of the amino group, the formaldehyde is liberated, and this is probably the reason for the reactivation observed by Ross and Stanley (1938). Fraenkel-Conrat (1954) who favors the structure  $R-N=CH_2$  for the formaldehyde complex, found that the virus ribonucleic acid had 2-7 % of formaldehyde bound, presumably to the primary amino groupings of adenine, cytosine, and guanine, in a relatively unstable manner, and he suggested that this was the reaction which caused the inactivation. This value indicates that most of the amino groups of the nucleic acid are involved in links with formaldehyde. This substitution has the effect of shifting the absorption maximum of the nucleic acid some 3-5  $m\mu$  toward the visible region.

#### *L. The Nucleic Acid of the Tobacco Mosaic Virus*

As has already been mentioned, the nucleic acid of the tobacco mosaic virus was first isolated by Bawden and Pirie (1937a) by a number of methods, including the denaturation of the virus protein by means of heat in the presence of salts, by the action of pyridine on the virus, and by the action of strong acetic acid on the virus, an action which has been shown recently to dissolve the protein in a relatively undenatured form. These authors found that the main constituents of the virus were protein and nucleic acid, and they made the important observation that the latter, which is of the "yeast" type or, as it is now called, ribonucleic acid, was relatively large compared with yeast nucleic acid, which at the time was prepared by an alkaline treatment which is now known to involve extensive degradation of the compound.

Some time later Sreenivasaya and Pirie (1938) prepared the nucleic acid by dissolving away the protein in a solution of sodium dodecyl sulfate.

The structure of the virus nucleic acid was first investigated by Loring (1939), who had an immense amount of virus available for this purpose. He found that it had the purine bases, adenine and guanine, and also the pyrimidines, cytosine and uracil. He also made the interesting observation that the uridylic acid was apparently an isomeric form of that reported in yeast nucleic acid. It is now known, of course, that yeast uridylic acid is a mixture of two isomeric substances, namely, uridine 3'-phosphate and uridine 2'-phosphate, and it is possible that Loring had isolated these in different proportions from those reported for the yeast nucleic acid derivative.



The physical properties of the nucleic acid were further investigated by Cohen and Stanley (1942), who prepared their material by the heat denaturation method, and they concluded that the nucleic acid was vastly larger (a molecular weight of 300,000) than yeast nucleic acid, which at the time was thought to have a molecular weight of about 1200. They also found that the virus nucleic acid was relatively easily degraded to smaller molecules.

When chromatographic methods for the analysis of the nucleic acids were developed, the composition of the nucleic acid of various strains of tobacco mosaic virus was reinvestigated. Using paper chromatography, Markham and J. D. Smith (1950) showed that the sugar in the nucleic acid was, as expected, ribose, a finding later confirmed again by the same method, and by the isolation of ribose as the di-*n*-propyl mercaptal by MacDonald and Knight (1953). The proportions of adenine, guanine, cytosine, and uracil were found to be 1.17 : 1.05 : 0.71 : 1.06 on a molar basis; this has been confirmed by a number of workers. (Knight, 1952; Schramm and Kerékjártó, 1952). It was also found that the base ratios of a number of strains of this virus were very similar, if not identical. The actual structure of the nucleic acids must, of course, differ in some way, but this difference may be reflected in a very minor way in the actual proportions of the bases. (Markham and J. D. Smith, 1950; Reddi and Knight, 1956). The only information as to the *arrangement* of the bases has been supplied by Reddi (1957b), who found that the nucleic acid of the "masked" strain of the tobacco mosaic virus had many more pyrimidine residues which could be released as mononucleotides by pancreatic ribonuclease, than had the other strains which he investigated. If this result is not due to a chance contamination of the virus nucleic acid with another enzyme, his findings would indicate a *very* large difference in the nucleotide sequence in the masked strain of the virus.

The apparent constancy of the composition of tobacco mosaic virus nucleic acid has been questioned by Commoner and Basler (1956) who have obtained results which suggest that there are vast variations in the base ratios at different times after the plants were infected with the virus. The significance of these claims is in some doubt, because the methods by which the results were obtained were novel and apparently had not been developed adequately. There is also a certain amount of internal evidence which suggests that the method of hydrolysis used was not sufficiently quantitative to make the results particularly meaningful. It would also appear likely that other workers would have noticed fairly large fluctuations in their analytical values, and there is every indication that this is not so.

The nucleic acid of this virus, like that of the turnip yellow mosaic virus, is evidently synthesized through compounds having a nucleoside 5'-phosphate structure (possibly the pyrophosphate or triphosphate) because phosphorus labeling of the 5'-nucleotides proceeds unevenly when virus is grown in the

presence of radioactive orthophosphate (Staehelin, 1958). The rate of labeling increases in the order cytidylic acid, uridylic acid, guanylic acid, and adenylic acid. When the virus nucleic acid is hydrolyzed to give the nucleoside 2'- and 3'-phosphates, the label is uniformly distributed. These effects are, of course, only observable for a short time after giving the  $\text{P}^{32}\text{O}_4^{3-}$ .

### *M. The Quantity of Nucleic Acid in the Virus*

When the virus is denatured by heat, it appears that the nucleic acid is liberated as one piece. The exact amount of nucleic acid present in the virus particles is a matter of some doubt, but a series of analyses by Black and Knight (1953) on seven strains suggests that the most likely phosphorus content is 0.51 %. This value is not incompatible with most of the other analytical figures which have been reported (the main source of error is in the determination of the actual weight of the virus used for the P estimations); as the average residue weight of the nucleotides is 319, the nucleic acid content of the virus would be 5.27 %, expressed as the free acid. There is some reason to suppose that the nucleic acid may exist as the free acid in this virus, because it is necessary to have salt present in order to separate the two components of the virus. In this case one would have to assume that, as in fish spermatozoa, the phosphate groups were neutralized by the arginine-guanidino groupings, and when the dissociation occurred the salt satisfied the charge requirements. The amount of free nucleic acid liberated would then be increased by the amount of cation which combined with it. Thus if the potassium salt were formed, the percentage would appear to be 5.7 %. With the sodium salt this would be 5.64 %. It is, however, very likely for reasons other than purely esthetic ones that the actual phosphorus percentage should be somewhat greater—about 0.546 %. The X-ray analysis of the virus shows that the nucleic acid is intimately associated with the protein subunits, buried in them, in fact, and it is therefore more or less imperative that the structures of the protein and the nucleic acid should be closely related. It is probable because of this that the number of phosphorus atoms is an integral number per protein subunit, and the most likely integral number is 3 (there is a slight possibility of there being 7 P atoms per 2 subunits, but for various reasons this would seem very unlikely). The quantity of free ribonucleic acid would then be about 5.64 % (this value will vary according to the estimate of the actual protein subunit size).

Hopkins and Sinsheimer (1955) actually measured the molecular weight of the ribonucleic acid, as liberated in salt solution by heating. The value which they got was 1.7 million compared with the value of 40 million obtained for the whole virus by the same method. The value anticipated would be about 2.5 million per 40 million as the sodium salt, or if one takes the rather

low phosphorus content of the virus found by these authors (0.45 %), it should be about 2 million. This discrepancy might well be due to the partial breakdown of the nucleic acid during its isolation. For example, Ginoza (1958), examining the stability of the virus nucleic acid to heating, as evidenced by its ability to cause infection, found that it was indeed very unstable, although his results do not preclude the possibility of the inactivation being due to a heat-stable enzyme, like plant ribonuclease, which was breaking down his nucleic acid, rather than a purely chemical type of hydrolysis as he assumed.

The possibility that the nucleic acid might be linked to the virus by very weak covalent bonds such as tertiary phosphate ester links is one which has attracted much attention. It does seem very unlikely, now, that such linkages can occur. Koshland *et al.* (1957) degraded the virus in the presence of  $\text{H}_2\text{O}^{18}$  by means of detergent and found no  $\text{O}^{18}$  in the ribonucleic acid released.

#### *N. The Effect of the Host on the Virus*

Many plant viruses are adapted to parasitize more than one host. Tobacco mosaic virus is no exception, and a considerable time ago it was thought of some interest to determine whether the virus was modified in any way by the association with the host on which it was growing. It is well known, for example, that in the case of other plant viruses a change of host may cause a permanent change in the symptom pattern produced by the virus, and while it was originally thought that this type of change was a host-induced modification, it is now fairly certain that the host acts by selecting mutants of a type which are more suited to multiplication on it. This type of change is, of course, certain to be reflected in a change in the chemical composition of the virus, although it may be so small as to escape detection. Similarly, chemical changes might be produced without an appreciable change in the symptom pattern.

The first attempt to determine whether there were any host-induced variations in a virus were made by Loring and Stanley (1937), who compared tobacco mosaic virus grown in tomato plants and in tobacco plants. Apart from minor differences in the physical properties, which are not altogether unexpected these days, these workers found little difference between their preparations. What chemical analyses were made were limited to elementary analyses, and it is quite evident that only very gross differences could be detected by the techniques used. Moreover, the two plants used in the investigation were closely related botanically, so that the system was not the most likely one to show up any differences due to the host.

A much more convincing system was studied by Gaw and Stanley (1947), who compared two distinct viruses, the "type" tobacco mosaic virus and the ribgrass virus, grown on tobacco and on *Phlox drummondii*, a plant only

distantly related to tobacco. The physical properties investigated did not differ greatly with the host, while the chemical properties studied were somewhat limited. For convenience in analysis the proportions of only 3 amino acids, namely, tyrosine, phenylalanine and tryptophan, were estimated. As it happens, the proportions of these amino acids differ between the two viruses, but they are not modified by varying the host. (In fact, the only differences which look particularly significant in this work are in the phosphorus, and hence nucleic acid, content of the viruses, which was greater when they were grown in *Phlox* by some 20 %.) It is, perhaps, unfortunate that only these 3 amino acids of the 18 or so amino acids present in the virus were studied. The choice was, of course, dictated by ease of analysis, but as any difference which might be expected might be a small one, and possibly involving only a small number of amino acids or even of one amino acid, it is more likely to be detected by a complete analysis which includes, in particular, those amino acids present in very small amounts. These are, for example, histidine, lysine, cysteine, and, of course, tryptophan, the values for which obtained in this work were rather higher than one might expect from more recent determinations.

In spite of the fact that the sample used was so small, and that only two strains of the virus were studied, the work done on *Phlox* and tobacco has caused nearly general acceptance of the dogma that the protein of virus has a definite composition which is independent of the nature of the host on which it was grown. This may indeed be so, but it is quite evident that this matter should be reinvestigated using modern techniques.

There is some evidence that the host may in fact modify the virus which is parasitizing it. A short time ago Bawden (1958) described a virus, related to the tobacco mosaic virus, which was found growing on a leguminous host, the cowpea, in Nigeria. This virus, unlike ordinary strains of tobacco mosaic virus, was able to parasitize bean (*Phaseolus vulgaris*) plants systemically, as well as tobacco, and when grown on the former host, it gave rise to very small and characteristic lesions on *Nicotiana glutinosa*. On transfer to tobacco, the virus changed its character, and gave larger lesions on the test plant, more like those of the type virus. These changes are reversible, and are accompanied by a definite change in the amino acid content of the protein, the most spectacular manifestation of which is the presence of histidine in the virus grown on bean plants and its absence in the virus grown on tobacco. This variation in the histidine content is compensated by a complementary variation in the lysine content of the protein.

The most probable explanation of this phenomenon is that the cowpea virus has a high mutation rate both from the tobacco type to the bean type and back again, and that the host plant dictates the type which is predominant. There is no question of the cowpea virus being a mixture of two distinct



viruses, because the isolation of the experimental material was performed at least as carefully as was the isolation of any other plant virus, and it certainly originated from a single lesion. There is also little possibility of the histidine being a terminal grouping on the polypeptide chain which is removed by specific enzyme systems in the tobacco plants, because by altering the growing conditions it is possible to maintain the "bean type" characters in virus grown in tobacco. This may be accomplished by growing the tobacco plants at a somewhat elevated temperature (30°C.). The yield of virus at this temperature is rather low.

Apart from the purely chemical differences noted above, the two forms of the cowpea virus have markedly different characteristics as determined in other ways. The immunological relationships are fairly remote, the electrophoretic mobilities are different, and, most striking of all, the X-ray diffraction patterns exhibit differences which are greater than those between the type strain and cucumber virus 4 (Holmes and Franklin, 1958). When one considers that cucumber virus 4 has been thought by some not to be a strain of the tobacco mosaic virus at all (Knight, 1954), this is all the more surprising.

### *O. Strains of the Tobacco Mosaic Virus*

It is now evident that what we recognize as a typical tobacco mosaic virus by its symptomatology is one of a number of strains of this type of virus which have established themselves successfully in commercial tobacco crops. Whether the virus is primarily a parasite of the tobacco plant is a matter of some doubt. Certainly the related ribgrass virus of Holmes (1941) is widespread both in America and in Europe (Harrison, 1956), and it has been suggested that the ribgrass (*Plantago lanceolata*) was introduced to eastern United States as a weed by the white settlers. It is possible, therefore, that the virus originally met its present host quite recently, just as it appears likely that, in Europe, the bulk of the tobacco mosaic type of viruses affecting the tomato crops originated from smoking tobacco which was infected with tobacco mosaic virus; in the small time in which such an association has been formed, the tomato mosaics have adapted themselves to tomato to such an extent that they are only transmitted with some difficulty to tobacco, in which they usually only produce a localized infection.

There are also strains of tobacco mosaic virus which infect numerous leguminous plants in the tropics: one, which has already been mentioned, occurs in cowpea, and another is found on Bombay hemp (*Crotalaria*) (Raychaudhuri, 1947); but from a scientific point of view the most interesting of these odd strains, and possibly the most divergent, is the cucumber virus 3, and its yellow mutant, cucumber virus 4.



### 1. *Cucumber Virus 3 (and 4)*

The viruses called by these odd names were originally known in England as common cucumber mosaic, a name now given to the aphid-borne cucumber mosaic virus, and they were evidently quite widespread. As a disease of economic importance cucumber virus 3 has almost vanished, although an epidemic was caused by it in Denmark quite recently (H. Rønde-Kristensen, private communication). In England and in America it is only a curiosity maintained among laboratory stocks.

Cucumber virus 3 is very limited in its host range, being confined to a few cucurbitaceous plants. It was only after its purification by Bawden and Pirie (1937b) that it was realized to be related to the tobacco mosaic virus. In physical properties this virus resembles the tobacco mosaic virus in almost every respect. The only gross difference lies in that the interparticle spacing observed by X-ray diffraction is slightly smaller than in the tobacco mosaic viruses (Bernal and Fankuchen, 1941a). As it is now known that this distance is not necessarily related to the actual width of the particles, and as the X-ray diffraction patterns obtained from the oriented virus resemble those of the type tobacco mosaic virus closely (Franklin, 1956b), there is every reason to suppose that the viruses are very similar. Although tobacco mosaic viruses do not in general infect cucumber plants, it is possible to show an interference in infection between cucumber virus 3 and tobacco mosaic virus on cucumber cotyledons (Rochow, 1956). This is a criterion of fairly close relationship between viruses, while the serological relationships of the two viruses are marked.

Cucumber virus 3 is unique among tobacco mosaic type viruses in that it contains no sulfur and hence has no cysteine or methionine. It also has no histidine (Knight, 1942, 1949, also Knight and Stanley, 1941). The tryptophan content is very low, being only one-quarter of that in the tobacco mosaic virus (Knight, 1947). The implications of this observation have been discussed earlier, and it appears that the subunit of the protein part of the virus is twice the size of the (apparent) subunit in the tobacco mosaic virus.

The nucleic acid has an appreciably different base ratio (adenine, 1.04; guanine, 1.03; cytosine, 0.73; and uracil, 1.19) from that of the type virus. The predominant amino acid released by carboxypeptidase from the protein is alanine (Knight, 1955a), and not threonine, which is found in a number of other strains of the latter virus (most of which were in fact very closely related, having been isolated from laboratory stocks). Largely on the basis of these observations, Knight (1955b) has suggested that the virus should not be regarded any longer as one of the tobacco mosaic viruses. This is, of course, purely a matter of definition or even of personal taste. The virus is so obviously related biologically and physically to tobacco mosaic viruses that the reasons for excluding it would seem rather arbitrary. It is interesting

to note, too, that the N-terminal end of this virus is also blocked by an acetyl group, and so the "N-terminal" sequence is somewhat reminiscent of that of the tobacco mosaic virus. It is reported to be (for Cucumber 4) *N*-acetyl alanine, tyrosine, asparagine, proline, isoleucine, threonine, serine—(Narita, 1958a).

## 2. Other Strains

The ribgrass virus has been discussed earlier in this section, while chemical differences existing between strains of tobacco mosaic virus are dealt with elsewhere.

## VII. THE TOMATO BUSHY STUNT VIRUS

This virus, which has some claim to fame as being the first virus to be crystallized in the form of three-dimensional crystals, was originally isolated from a tomato plant which was found infected under natural conditions (Smith, 1935). This one isolate is the only one which has been worked with by workers in various countries, largely because the disease was never again recorded until a few years ago, when it was found to be widespread in Italy (Gigante, 1955). This fortunate occurrence, the isolation of a stable virus from a single source, has had the happy result that all workers have been dealing with one virus, or at the least, virus which came from one original source. Of course, there is the difficulty that only one strain has been available for investigation, but this trouble has now been bypassed to some extent with the availability of the Italian isolates, which seem to differ somewhat from the original type strain in their composition.

Although from its name one might imagine that its preferred host was tomato, a much more suitable host for propagation is *Datura* (*tatula* or *stramonium*) (Stanley, 1940), because the latter is more readily infected and is less susceptible to chance infection with tobacco mosaic. It may also be grown on cucumber, tobacco, beans, cowpeas, and a number of other plants. These last plants give only local infections under normal conditions, so that the yield is not high.

### A. Purification

The original purification was made by Bawden and Pirie (1938b) who clarified their sap (from tomatoes) by heating to 60°C. This step, which was known from the early observations of Smith (1935) to cause the inactivation of the virus, resulted in a great loss of infectivity, but little if any loss of material. This very fact, in these days when virus nucleic acids are being used for transmission, makes one wonder what can possibly happen to the virus under these conditions to cause its inactivation. Indeed this virus loses

its infectivity fairly rapidly at 37°C., but apparently without any gross change in its chemistry. It is quite evident that our ideas about virus structure and infectivity are oversimplified.

The next purification step was precipitation with ammonium sulfate (200 gm./liter), followed by successive precipitations with this salt. The virus is deposited on the addition of salt as an amorphous precipitate, which is more soluble in the cold than at room temperature. This fortunate property enables one to centrifuge a solution of the amorphous material in a refrigerated centrifuge and remove some of the impurities. On further standing, the crystalline form of the virus separates out as beautiful rhombic dodecahedral crystals (Fig. 9). These are very slow to dissolve in cold water, and may be washed if necessary. They can be dissolved very readily in 50 % (v./v.) ethanol, but will dissolve in water after a time. (The solubility is large. It is only the rate of solution that is small.)

Stanley (1940), when he came to investigate the virus, elected to omit the heating step, which caused so much loss of infectivity, and used *Datura* as host. He also used high-speed centrifugation rather than salting out for the isolation, although the last step has no obvious advantage, because it is usual to finish the preparation by crystallizing the virus.

The yields are somewhat variable but are of the order of 50 to 100 mg./liter of sap.

### *B. Chemical Properties*

The virus contains about 15 % of ribonucleic acid ( $P = 1.58$  %), which has been analyzed by Markham and J. D. Smith (1951b) and de Fremery and Knight (1955). The relative proportions of adenine, guanine, cytosine, and uracil are 25, 28, 21, and 25 on a molar basis. Analyses of the nucleic acid had to be made on the whole virus, because the former is very tightly bound, presumably because of the large numbers of electrovalent links between the protein and the nucleic acid (although, as in turnip yellow mosaic virus, the basic amino acids present are insufficient to neutralize all the phosphate groups).

The protein of the virus has been analyzed by ion exchange chromatography by de Fremery and Knight, who actually analyzed three isolates which had slightly different symptomatology but had almost identical compositions. The results obtained, in grams of residues per 100 grams of virus, were: alanine, 4.8; arginine, 5.8; aspartic acid, 9.3; cysteine, 0.6; glutamic acid, 5.0; glycine, 4.1; histidine, 1.2; isoleucine, 2.8; leucine, 9.0; lysine, 3.1; methionine, 0.9; phenylalanine, 3.8; proline, 2.7; serine, 5.5; threonine, 8.1; tryptophan, 0.6; tyrosine, 3.3; valine, 7.2; and amide, 1.16. Estimated colorimetrically, arginine was 6.4. The glycine figure contains an

uncertainty because of the glycine arising from the breakdown of the purines of the ribonucleic acid.

Caspar (1956a) has pointed out that the tryptophan, cysteine, methionine, and histidine are in the proportion of 1 : 2 : 2 : 3 residues in a molecular weight of 25,000 to 28,000, which he proposes as the size of the protein subunit of the virus.

### *C. Physical Properties*

The virus dissolves in aqueous solvents to give opalescent solutions which are isotropic. The isoelectric point of the virus is at pH 4.1 (McFarlane and Kekwick, 1938), and the virus is soluble over a wide range of pH's. The ultraviolet absorption shows a peak at 262 m $\mu$ , and the extinction of a 0.1-mg./ml. solution is 0.52 for a 1-cm. layer (Bawden and Pirie, 1938b).

The molecular weight from light scattering is 9,000,000 (Oster, 1946); this is in good agreement with results obtained by other methods, which are summarized by Markham (1953b), and include a direct particle count (Williams and Backus, 1949). The sedimentation coefficient is 131 *S* (Laufer and Stanley, 1940), the earlier value of 146 *S* given by McFarlane and Kekwick (1938) having been in error owing to a defect in the hydrogen-cooling supply in the Svedberg equilibrium centrifuge used for the measurement (R. A. Kekwick, personal communication). Much of the early work on the size and molecular weight of this virus is discussed at length by Markham *et al.* (1942).

Bushy stunt virus was one of the earliest viruses to be investigated by X-ray crystallographic techniques. The original experiments were made by Bernal *et al.* (1938); (Bernal and Fankuchen, 1941b) who used powders of small crystals, and examined them both wet and dry. The wet crystals gave reflections which were consistent with a body-centered cubic lattice of side 394 Å. On drying the crystals shrink, and the crystal spacings also shrink; in the dry crystal the only spacing obtained indicated that the cell size had decreased to a 318 Å cube. This interesting work indicates that the virus is itself hydrated and shrinks on drying, because the average diameter of the particles deduced from the diffusion coefficient of Neurath and Cooper (1940),  $1.15 \times 10^{-7}$  cm.<sup>2</sup>/sec., is 37 m $\mu$ . A body-centered cube having rigid spherical particles of this size in point contact would have an edge of 42.7 m $\mu$ , so that the particles must be in contact over a flattened area and have an effective diameter 92.5 % of that of the equivalent spheres. (This is what one would expect if the particles were polyhedral. The smallest diameter of an icosahedron is about 81 % that of a sphere of equal volume.) The anhydrous particles having a molecular weight of about 10 million, if they were the same shape as the hydrated particles, would give a cube of side 310 Å. The agreement between these figures is a strong indication that the particles



swell reversibly on wetting. The disorder, evidenced by the loss of reflexions on drying the crystals, is paralleled to some extent by a loss of infectivity of the virus by incautious drying. Infectivity may be retained on drying by very slow drying over solutions of relatively high vapor pressure, such as saturated zinc sulfate in water.

Stationary single crystal X-ray diffraction measurements were made by Carlisle and Dornberger (1948), who obtained similar but slightly smaller cell sizes, the wet cell being 368 Å and the dry one 314 Å. It is, of course, possible that the wet cell size will vary with the suspending fluid, the powder photographs having been taken with the crystals fully immersed in the mother liquor, whereas single crystals are usually drained.

The X-ray structure has been reexamined more recently by Caspar (1956a), using single crystal precession photographs, and he deduced that the virus particles themselves possessed cubic symmetry, and were built up from 60 equivalent subunits, or a multiple of 60 subunits, as has been suggested by Crick and Watson (1956) from theoretical considerations. As mentioned earlier, this idea is compatible with the analytical data which have been published. Caspar thinks that the number of protein subunits involved is probably 360.

Bushy stunt virus crystallizes in other forms in the presence of substances such as heparin (Cohen, 1942), but these crystals have not been examined in any detail so far. Superficially, they look as if they do not belong to the cubic system.

### VIII. THE TOBACCO RINGSPOT VIRUS

This virus, which is a common disease agent of tobacco and a number of other plants, receives its name from its characteristic of causing the formation of concentric rings on many of its hosts (Fig. 10). This tendency to form rings is one of the characteristics of plant viruses, but the mechanism involved is uncertain.

In 1939 Stanley reported the isolation of tobacco ringspot virus from Turkish tobacco plants (Stanley, 1939). This virus is present in such plants in small quantity only, and the isolation was performed by high-speed centrifugation because much of the activity of the virus is lost when ammonium sulfate is used for the precipitation. The material which was obtained was fairly unstable at room temperature, but was stable at 4°C. It contained a very large amount of nucleic acid, estimated from the phosphorus content as 40 %, a substantial proportion of which was isolated as (somewhat degraded) ribonucleic acid by the method of Johnson and Harkins (1929). The virus had a sedimentation coefficient of 115 *S*, and appeared to be essentially spherical from the viscosity and from the lack of anisotropy



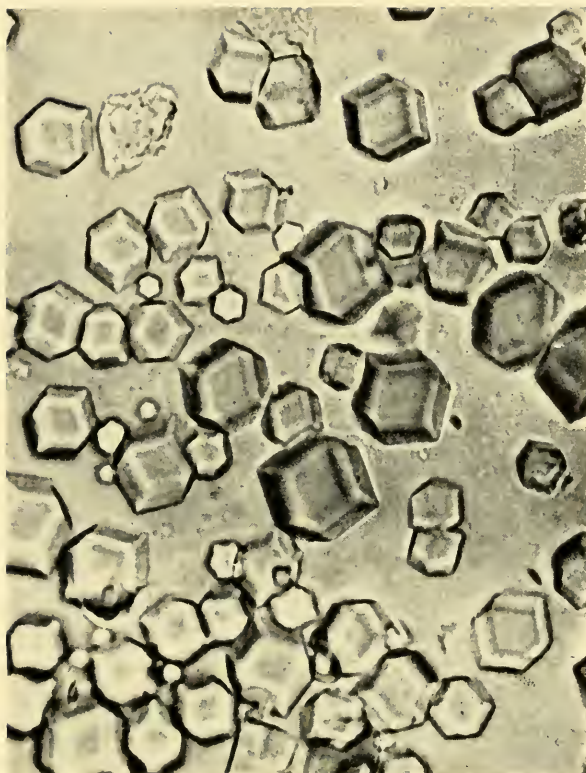


FIG. 9. Rhombic dodecahedral crystals of tomato bushy stunt virus.



FIG. 10. Tobacco plant showing symptoms of tobacco ringspot virus infection.



FIG. 11. Chinese cabbage plant with turnip yellow mosaic virus. The local lesions can be seen on the inoculated leaf (bottom left).

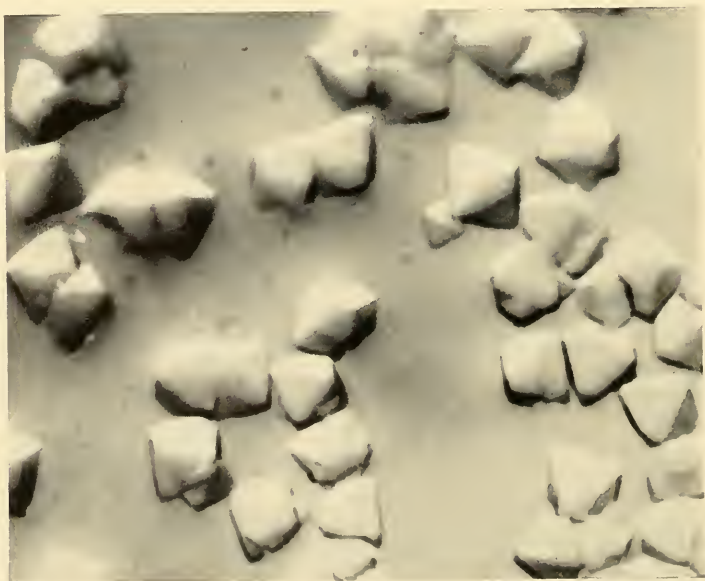


FIG. 12. Crystals of turnip yellow mosaic virus from ammonium sulfate.

of flow of the solutions. An interesting observation was that much of the nucleic acid was lost when the virus suspensions were precipitated with ammonium sulfate, but as it now appears that the preparations were not pure and were contaminated with nucleic acid-containing particles of plant origin, it is not altogether certain whether the two phenomena are connected.

The status of the tobacco ringspot virus remained much the same until quite recently when the virus was reexamined by Steere (1956). Steere found that the most suitable source of virus for purification was the cotyledons of the Caserta squash (*Cucurbita pepo*), which yield some 45 mg. of virus per liter of expressed sap. He also introduced a novel method for the clarification of the sap, in which he used a chloroform-*n*-butanol treatment, a treatment similar to that used by Morton (1953) in his experiments on extracting enzymes from lipid-containing materials. The aqueous phase so obtained was subjected to three successive sedimentations at high speed out of 0.01 *M* phosphate buffer of pH 7. The centrifuge pellets were resuspended in water before the phosphate was added, however, because the pellets do not resuspend readily in the buffer. Controls run using healthy plants did not yield any of the material.

### *A. Physical Properties*

Solutions of the virus containing 2 % or more, when stored at 4°C. in phosphate buffer (0.1 or 0.01 *M*) gradually deposit small crystals, which evidently belong to the cubic system. Some appear to be derived from octahedra, others from dodecahedra. In the crystalline state the virus gradually loses its infectivity.

Examined under the electron microscope, the ringspot virus particles appear to be polyhedral in form, about 26 m $\mu$  in diameter, especially if freeze-dried, and show both hexagonal and pentagonal peripheries, indicating that the particles are probably icosahedral in form (Kaesberg, 1956).

As with most other plant viruses the number of particles required to form a single local lesion (in cowpeas) is large, being of the order of  $10^5$ , and as the preparations contain some 5 % of material having a lower sedimentation coefficient (89 *S*) than that of the remainder (116 *S*), experiments were carried out to check that the infectivity was really associated with the bulk of the material. This was found to be so. Oddly enough the virus preparation had only one electrophoretic component ( $11.3 \times 10^{-5}$  cm./sec./volt/cm. at pH 7 in 0.05 *M* phosphate). This would suggest that the more slowly sedimenting component was similar to the nucleic acid-free virus proteins found in other virus preparations.

### *B. Chemical Properties*

The virus has not been investigated very thoroughly as yet. The phosphorus content is about 3.1 %, indicating that the virus has some 35 % of nucleic acid, presumably of the ribose type.

## IX. THE TURNIP YELLOW MOSAIC VIRUS

There are several viruses which cause mosaics in turnip; one of these, which has many interesting characteristics, causes an extremely bold mottling. This virus, the turnip yellow mosaic virus, is fairly widespread in Europe, and causes serious disease in turnips, cauliflowers, savoy cabbages, and other cruciferous plants of economic importance. It is a virus which is present in the host plants in fairly large concentrations, as much as 4 grams per liter of sap having been isolated under somewhat exceptional circumstances, and it is spread about under natural conditions by the turnip flea beetle (*Phyllotreta* sp.). This method of spread is in itself unusual, and is dependent upon the organization of the digestive system of the beetles coupled with the resistance of the virus to digestion (Markham and K. M. Smith, 1949).

A number of strains of the virus are known, all having similar effects on the host plants. One complication which has not been resolved (and which has not been published before) is that all stocks which are being investigated as the "type" strain of this virus at the moment may have originated from a different isolate from that originally described. This was due to a mixing of two isolates of the virus by accident in 1951. The present cultures resemble the original isolates closely, but may in fact differ slightly.

Besides the "type" virus, cultures of other isolates are maintained in England, but have not been investigated thoroughly so far.

### *A. Purification*

The virus is best cultured on Chinese cabbage or turnip plants, in which it produces its characteristic yellow mosaic (Fig. 11). The rate at which the virus multiplies is slow, and appears to increase as the plants begin to suffer from malnutrition. Consequently the plants should be grown in small pots and be allowed to become pot-bound. They are harvested about 2 months after infection, and then ground, and the sap expressed.

The sap is clarified by the addition of 300 ml. of 90 % (v.v.) ethanol to each liter of sap, the flocculent precipitate of plant proteins spun off, and the supernatant liquid, which is yellow and slightly opalescent, has a half volume of saturated ammonium sulfate in water added. Crystallization of the virus begins in a few minutes, and is complete in 4 or 5 hours. At the same time, strongly birefringent crystals, possibly of calcium sulfate, are



also found, but these are insoluble in water, so that the pellet of crystals obtained on centrifuging may be extracted with water and reprecipitated as crystals.

The virus can, of course, be prepared by centrifuging the clarified sap, but this method has no obvious advantage over the salt precipitation method, which can, if necessary, be scaled up to the tens of liters by using a Sharples centrifuge both for clarification and for collecting the crystals.

The original method for purifying the virus (Markham and K. M. Smith, 1946) depended upon an alcohol precipitation step which is only feasible under winter temperature conditions in England, but which might be successful in a cold room in hotter climates.

### *B. Further Purification*

Under normal conditions three or four recrystallizations of the virus from ammonium sulfate suffice for the purification. Crystallization follows smoothly after enough salt has been added to make the solution slightly cloudy, and it is virtually impossible to prepare the virus in an amorphous form. The crystals are octahedra (Fig. 12).

The virus is fairly resistant to enzymatic attack, and pancreatic extract has been used for cleaning up preparations, but this is always accompanied by the absorption of enzymes on to the virus particles. Another method which may be employed for the purification is crystallization out of ethanol solutions. A solution of the virus in water, containing about 5 mg./ml. and having a trace of a salt (such as ammonium sulfate) present, is cooled to 0°C. Absolute ethanol (0.3 vol.), also cooled, is added with stirring. The solution is then acidified by adding a few drops of a solution (ethanol 20 parts, acetic acid 10 parts, water to 100 parts) until the virus solution begins to become cloudy.

The virus then will crystallize out in the cold in the form of bunches of fine birefringent needles, or occasionally as flat plates, which are exceedingly thin. When centrifuged down, the crystals dissolve readily in 0.1 *M* Na<sub>2</sub>HPO<sub>4</sub>, or on the addition of NH<sub>3</sub> to a suspension in water. Contaminants may then be spun off. This crystallization takes place at about the isoelectric pH of the virus (3.7).

### *C. Properties of the Preparation*

The virus dissolves readily in water, and is soluble over a wide pH range. The solutions are opalescent and isotropic and have a characteristic ultra-violet light absorption. The optical density is about 0.7 for 1 cm. at 0.1 mg./ml. but varies slightly (Fig. 13).



The virus may be crystallized out from aqueous solutions by the addition of various salts, such as magnesium sulfate, sodium thiosulfate and even sodium chloride. It is denatured by heating under a number of conditions [it is extremely labile in phosphate buffer at 50°C. (Matthews, 1957)], and the nucleic acid is liberated. A much better way of liberating the nucleic acid,

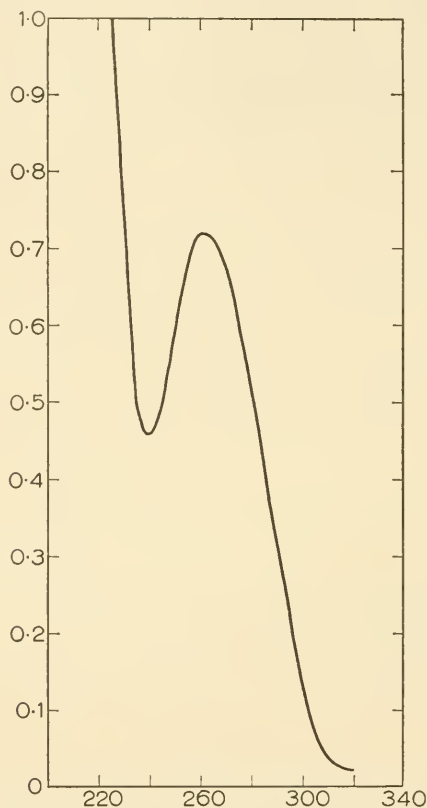


FIG. 13. Absorption spectrum of turnip yellow mosaic virus plus the accompanying "top" component, at a concentration of 0.1 mg./ml., recorded on a Cary No. 14 spectrophotometer. Compare the large peak to trough ratio of the spectrum of this small spherical virus with that of the tobacco mosaic virus (Fig. 8).

however, is by the addition of half a volume of ethanol to the solution. In the presence of salt the protein flocculates and leaves the nucleic acid in solution. The nucleic acid may then be precipitated by the addition of more ethanol, or by acidifying with acetic acid. The ease of separation of the nucleic acid of the type virus is not paralleled in all the strains, probably because the type strain contains relatively little arginine compared with the large amount of nucleic acid present.



FIG. 14. Electrophoretic diagram of the turnip yellow mosaic virus proteins at pH 5.3S. A, ascending boundary; B, descending boundary. Concentration, 5 mg./ml.

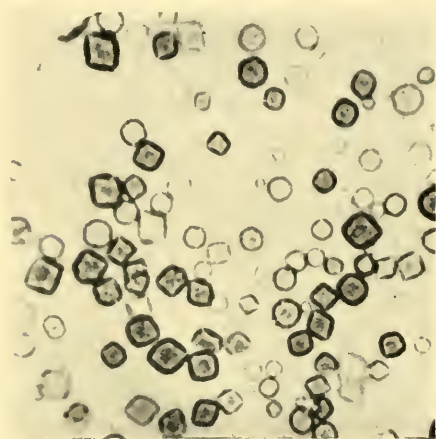


FIG. 16. Ultraviolet photograph (265  $m\mu$ ) of crystals of turnip yellow mosaic nucleoprotein (dark) and the associated protein (light). The two substances were crystallized separately before they were mixed (contact print enlarged about  $\times 200$ ).



FIG. 15. Sedimentation diagram of the turnip yellow mosaic virus showing the two components (5 mg./ml., 24,000 r.p.m.)



*D. The Two Components*

One of the features of the turnip yellow mosaic virus which has caused it to be of great interest to virus workers is that, unlike the virus preparations which had been obtained previously, this virus always gives rise to two kinds of proteins which appear to be very similar, and which are possibly identical, but only one of which is infectious. The way in which this was discovered is of some interest. The original preparations of the virus were made by the methods outlined above, and on examination by the usual methods were found to behave much as a virus might be expected to. The material was fully crystalline, had a nucleoprotein spectrum, and was highly infectious (2 infections out of 8 at  $10^{-9}$  gm./ml.). The preparation consisted of uniform spherical particles when examined electron microscopically. The phosphorus content was large, about 2.13–2.24 %, but varied in an inexplicable way from preparation to preparation. On examination on the Tiselius apparatus over a range of pH's only one boundary was visible and the isoelectric point was pH 3.75 (Fig. 14). The wet size of the virus was determined by diffusion and the diameter was 280 Å. It was with some surprise, therefore, that when it was examined in the ultracentrifuge, it was realized that two compounds were present (Fig. 15). These had sedimentation coefficients of 106 and 49 *S*; the latter accounted for about a quarter of the total material. For a time it was thought that the 49 *S* material was the virus and that the 106 *S* material represented a 4 : 1 aggregation of the former, but it later proved possible to isolate a few milligrams of the 49 *S* ("top") component by means of an ultracentrifuge partition cell. Fortunately this material was examined by a number of methods. The actual concentration was known to a close approximation from the area of the sedimentation pattern, and this was checked by quantitative serological precipitation. One milligram was set aside for crystallization, and yielded the usual octahedral crystals. One-tenth of a milligram was used for absorption spectrophotometry, when it was found that instead of having an optical density per cm.<sup>3</sup> at 260 mμ of about 0.7, it had a negligible absorption. The rest of the material was used for pentose estimation, which showed that little or no nucleic acid was present, and for infectivity measurements, which indicated that the material was not infectious.

Later it was found possible to prepare quantities of both components in a fairly pure condition largely by centrifuging. The methods employed are somewhat complex, and are discussed in full elsewhere. The examination of these preparations confirmed that there are, in fact, two substances, a nucleoprotein and a protein, produced by the virus infection. These resemble each other in a remarkable fashion. The points of similarity and of difference are summarized in the following:

### 1. *Points of Similarity*

The nucleoprotein (bottom) and protein (top) components resemble each other in many ways. They each contain about  $3 \times 10^6$  of protein (as molecular weight), and the amino acid compositions of these proteins are closely similar (Markham *et al.*, 1948a; Fraser and Cosentino, 1957). The two proteins electrophorese together, having isoelectric points of pH 3.75, they both crystallize as octahedra from salt solutions, and they will form mixed crystals. The diffusion coefficients are very similar, and the diameters of the hydrated particles are very closely alike (about 280 Å), as shown by the diffusion measurements, X-ray crystallography (Bernal and Carlisle, 1948), and X-ray scattering. The particles are essentially spheres as seen on the electron microscope. Both types of particle react with antiserum to the virus, and no differences can be detected by serological means.

### 2. *Points of Difference*

The nucleoprotein contains about 37 % of ribonucleic acid (as the free acid) and has an ultraviolet absorption maximum at 263 mμ. The optical density of a 1-cm. layer at 0.1 mg./ml. is 0.95. The protein component has an ultraviolet absorption maximum at 276 mμ, where the density of a 0.1-mg./ml. solution is 0.11. Although the crystals are similar in appearance, (Fig. 16), the crystals of the protein component float when centrifuged in a sodium thiosulfate solution of specific gravity 1.32, while the nucleoprotein crystals sink. The nucleoprotein has 3.77 % of phosphorus, while the protein has none. The specific volume of the nucleoprotein is 0.67, and that of the protein is 0.74. The molecular weights are  $5 \times 10^6$  and  $3 \times 10^6$ , respectively (Markham, 1951). The nucleoprotein is a much better antigen (inducing antibody formation). X-ray scattering shows that the nucleoprotein is essentially solid, and that the protein is hollow with an internal diameter of about 75 % of the external one (Schmidt *et al.*, 1954). The protein component also tends to collapse more on drying for electron microscopy (Cosentino *et al.*, 1956).

### 3. *The Interpretation of the Above*

By a study of the above it was easily seen in what way the two materials were related. The nucleoprotein component quite evidently consists of a shell of protein which encloses a core containing the ribonucleic acid. The ribonucleic acid is necessary for the infectivity of the virus particles, but it does not have much effect on the surface properties of the virus, which are essentially decided by the amino acid side chains on the protein surface.

The exact function of the "top" component is uncertain and considerable speculation has been wasted on the problem. One of the striking factors is that the top component is always present in every host, and the proportion



is essentially much the same regardless of the age of the infection and of the general cultural conditions. Matthews (1958) has made an intensive investigation of this problem and has found a ratio of two nucleoprotein particles to one protein particle (by number) over a wide range of conditions, and he has suggested that the top component is the empty shell from which the nucleic acid has emerged to give rise to two new virus particles (he actually says three, but this is probably an error). On the other hand, Jeener (1954) favors the idea that the top component is a precursor of the virus nucleoprotein. His reasons for this are that the top component is much more rapidly labeled when the host plants are exposed to  $C^{14}O_2$ , than are the virus nucleoprotein particles. These two observations are very difficult to reconcile, but a fuller and more systematic investigation of the carbon labeling may throw further light on the matter. It may prove that the top component is not a homogeneous population (in its origin at least), and contains both virus particles in the making and the shells of virus particles which have lost their nucleic acid.

There is no reason to think that the top component is an artifact produced during the isolation procedure, because it may be demonstrated in preparations which have been isolated in a number of different ways. It is also possible to take healthy plant sap, add the nucleoprotein to it in a pure form and reisolate without any top component being formed in the process (Markham, 1953a).

The crystals were first examined by Bernal and Carlisle (1948) who found that both top and bottom component crystals gave powder diagrams which fitted a face-centered cubic lattice with a unit cell of about 710 Å side. (The top component crystals have a rather larger unit cell.) This was, of course, much larger than could be filled with single virus particles, and a number of reflections were found to be missing, which suggested that the lattice was in fact a diamond lattice with 8 virus particles per cell, and an interparticle distance of 30.6 mμ. This was in agreement with small crystalline aggregates of the virus observed under the electron microscope by Cosslett and Markham (1948).

Some time later larger crystals became available and still photographs of these were taken by Bernal and Carlisle (1951). The unit cell of the nucleoprotein was found to have a 703-Å side, and the protein a 715-Å side; Bernal and Carlisle suggested that the presence of the nucleic acid caused the virus particles to be more compact by holding them together (this could be due to attraction between the negative charges of the nucleic acid acting on the interior of the virus shell, which probably has a large number of positive charges on it).

The problem was investigated somewhat later by Klug and associates (1957a,b) who actually used some of the same material as did Bernal and

Carlisle in their work. Using some large single crystals of the nucleoprotein, they took precession photographs, and found that the unit cell when viewed at *low* resolution appeared to be a body centred cubic cell of side 350 Å, and that the centers of the virus particles occupied the lattice points. This observation is of interest, inasmuch as it implies that the particles themselves must have cubic symmetry. The true unit cell is however 8 times the volume, and so has 16 particles per cell instead of the 8 which one would expect from the diamond lattice, which is rather full of spaces.

The fact that the 700-Å cells did contain 16 particles, and not 8 as deduced by Bernal and Carlisle, was confirmed in a direct way by measuring the ultraviolet light absorption of single virus crystals of known thickness, and comparing it with the known ultraviolet absorption spectrum of solutions of the virus. In this way it was shown (with the collaboration of Dr. P. M. B. Walker) that the unit cell contained  $18 \pm 3$  virus particles.

A satisfactory solution of the X-ray crystallographic anomalies seems to have been reached by the assumption that the crystal lattice consists of two interpenetrating diamond lattices in which the particles are orientated *alternately* at 90° rotation. This arrangement gives a pattern superficially similar to that of a 350-Å body-centered cubic cell, but it will be noted that the central particle of such a cell will be rotated by 90° with respect to those particles which (partially) occupy the cube apices. The symmetry of such a structure demands that the virus particles should be built up from at least 12 equivalent subunits, but the question of the particle symmetry has been pursued further by studying the top component crystals, where there is less possibility of interference by the scattering of the nucleic acid component, which contributes about half of the scattering of X-rays and which may well have different symmetry itself.

#### 4. *The Symmetry of the Top Component*

The top component has been studied mainly as a powder because large crystals are difficult to obtain. The patterns indicate that the particles are hollow spheres with internal and external diameters of 210 and 280 Å. There is an indication that the spheres are not uniform in their scatter, but that they have a periodicity of about 60 Å. A model of such a hollow sphere has been constructed, using 60 subunits of 60 Å diameter arranged in the form of a snub dodecahedron (Fig. 17), and this type of model would effectively satisfy the X-ray requirements. It would also mean that one might expect 60 chemical subunits (or a multiple of 60) in the protein component of the virus. This will be discussed later.

The fact that the virus particles seem to be built up of regular subunits is also suggested by electron micrographs made by Cosslett (1956) and by Steere (1957). These pictures do not, however, appear to show as many

subunits as one might expect from the studies of Klug *et al.* (1957), but the resolution required is at the limit of present-day techniques (Fig. 18). As an alternative to the snub dodecahedron a model based upon the icosahedron would have rather similar properties. It is also quite likely that the 60-Å subunit is itself composed of a number (probably 2) of subunits.



FIG. 17. Sketch of snub dodecahedron made up from 60 spherical subunits. This structure is proposed for the turnip yellow mosaic virus by Klug *et al.* (1957).

#### *E. Amino Acid Composition*

The amino acid composition was first investigated by paper chromatographic analysis (Markham *et al.*, 1948a), but the first quantitative analyses were made by Roberts and Ramasarma (1952) who employed microbiological assay methods. More recently Fraser and Cosentino (1957) have analyzed the protein component of the virus and of the top component by ion-exchange chromatography. The results of these analyses are tabulated in Table IV.

There are a number of discrepancies between these sets of data, the most striking one being the apparent absence of cysteine in the analyses made by Fraser and Cosentino. Analyses made in Cambridge indicate that cysteine is indeed present as found by Roberts and Ramasarma.

The tryptophan analyses made by the latter are also of interest, because they indicate a subunit size of about 20,000, a value which is consistent with analyses made on two strains of the virus having vastly different acid compositions and so far unpublished. The fine structure of the protein, which would have some 280 amino acids, is also under investigation.

An interesting consequence of these analyses is that the number of basic amino acids present in the virus is such that they cannot neutralize more than a fraction of the phosphorus atoms in the nucleic acid, so that the proportion of base present in the virus must be quite high. The nature of the basic substances present is also being investigated.

TABLE IV  
GRAMS AMINO ACID PER 100 GM. PROTEIN<sup>a</sup>

| Amino acid    | A    | B    | C    |
|---------------|------|------|------|
| Alanine       | 5.1  | 5.4  | 5.7  |
| Arginine      | 2.4  | 2.2  | 2.5  |
| Aspartic acid | 5.9  | 6.3  | 5.1  |
| Cysteine      | 0.0  | 0.0  | 2.50 |
| Glutamic acid | 7.7  | 8.0  | 9.6  |
| Glycine       | 2.5  | 3.8  | 2.9  |
| Histidine     | 2.2  | 1.6  | 2.1  |
| Leucine       | 8.5  | 8.6  | 10.3 |
| Isoleucine    | 7.4  | 7.4  | 10.8 |
| Lysine        | 5.0  | 5.0  | 10.7 |
| Methionine    | 2.1  | 2.1  | 2.85 |
| Phenylalanine | 3.5  | 3.6  | 3.8  |
| Proline       | 9.7  | 11.8 | 10.7 |
| Serine        | 6.3  | 6.7  | 8.1  |
| Threonine     | 11.3 | 12.2 | 15.1 |
| Tryptophan    | —    | —    | 1.14 |
| Tyrosine      | 2.0  | 2.2  | 2.45 |
| Valine        | 6.2  | 6.2  | 8.45 |
| Amide N       | —    | —    | 1.25 |

<sup>a</sup>A, top component; B, bottom component (Fraser and Cosentino, 1957); C, whole virus (Roberts and Ramasarma, 1952) of type strain.

### *F. The Nucleic Acid*

The ribonucleic acid of the turnip yellow mosaic virus constitutes some 37 % of the total weight (as the free acid) and thus has a "molecular weight" equivalent to 1,850,000. Attempts have been made to estimate the actual molecular weight of the nucleic acid by various methods, but none are unequivocal. Certainly the estimates made by the analytical estimation of end groups by Markham and J. D. Smith (1952c) are obviously in error, probably owing to contamination with plant ribonuclease.

The nucleotide composition of the nucleic acid is of interest because of its unusual distribution of bases (Markham and J. D. Smith, 1950). The bases are: adenine, 23; guanine, 17; cytosine, 38; and uracil, 22 residues per 100 residues. This composition is so unusual that it has defied all the attempts to fit it to any of the proposed schemes of ribonucleic acid structure.

The sugar was first shown to be ribose by chromatographic methods, and later by using ribonuclease (Markham and Smith, 1952a). This nucleic acid was in fact used as substrate in many of the experiments involved in the study of ribonuclease action.



The mild way in which the nucleic acid may be liberated from the virus, by treatment with ethanol of more than 34 % v./v., in the presence of salt, at neutral pH, and in the cold, is such that it should produce nearly native nucleic acid, provided that enzyme contamination can be prevented. The nucleic acid itself is probably much coiled inside the virus, and probably cross-linked by means of polyvalent bases, but on extraction it forms viscous solutions, probably because it extends when removed from its protein coat.

## X. THE WILD CUCUMBER MOSAIC VIRUS

The wild cucumber mosaic virus was isolated from a naturally infected wild plant by Freitag (1952) and has been investigated by Sinclair *et al.* (1957). Its general properties are very interesting, because it resembles in many ways the turnip yellow mosaic virus, with which, however, it does not appear to be related immunologically (P. Kaesberg, personal communication).

### A. Purification

The virus was grown on *Cucurbita pepo* (pumpkin) and was purified by alternate cycles of high- and low-speed centrifugation. When examined on the ultracentrifuge, it was found to have two components, which were separated by the use of an ultracentrifuge separation cell, and it was found that the slower sedimenting component was noninfectious.

Larger-scale separations of the two components were made using the density gradient method of Brakke (1953). Tests of the two zones obtained in a number of runs confirmed that the slower sedimenting component was noninfectious.

### B. The Two Components

The faster sedimenting or "bottom" component, which amounts to about three-fourths of the whole, has a typical nucleoprotein spectrum with a peak at about 260 m $\mu$ . The other or "top" component has a diffuse spectrum with no definite maximum, but it evidently does not contain nucleic acid.

Ultracentrifugation of the two components at pH 7.0 gave sedimentation coefficients of 54.4 *S* and 107.5 *S* extrapolated to zero concentration. At pH 8, however, only the top component peak was found, presumably because of the loss of the nucleic acid at this pH. This is a most startling observation, and one which should be of the utmost use in further work on this virus. The virus also loses its infectivity when exposed to this pH, as one would expect.

Electrophoretic examination showed that there was only one component over a fairly wide range of pH's. This is a further indication that the electrophoretic method is only of use in comparing the surfaces of large molecules. The isoelectric point of the virus is pH 6.6, which is unusually high for viruses.



The diffusion coefficient ( $D$ ) of the mixture has been measured approximately and has a value of about  $1.1 \times 10^{-7}$  cm.<sup>2</sup>/sec. This would give a particle diameter in the hydrated state of 38.7 m $\mu$ . This is probably an overestimate because X-ray scattering measurements gave a diameter of 284 Å for the bottom component (the corresponding  $D$  would be  $1.5 \times 10^{-7}$  cm.<sup>2</sup>/sec.). The top component gives an indication of being hollow, the external and internal diameters being 280 and 210 Å.

Seen on the electron microscope the particles are evidently near spheres, which when examined frozen-dried have a polyhedral appearance. If air-dried they collapse, the top component being less stable than the bottom component.

Yields and chemical analyses of the virus have not yet been given. It would appear that the material is rather scarce and difficult to deal with, but it is to be hoped that a more detailed investigation of this interesting virus will be made. In particular, the possibility of removing the nucleic acid by a relatively mild treatment would make the virus very useful for X-ray crystallographic studies, if crystals of the virus can be obtained (which should be possible).

## XI. THE TOBACCO NECROSIS VIRUSES

The tobacco necrosis viruses were first encountered in the glasshouses used for virus research in Cambridge in 1935 (Smith and Bald, 1935), where they were found affecting the roots of many of the experimental plants. When emulsions of such roots were inoculated on to the leaves of the plants, local necrotic lesions appeared after a few days (Fig. 19). It was characteristic of such lesions that they remained localized and did not cause systemic virus infection. The host range was enormous, almost all plants being susceptible. The origin of the virus was at the time obscure, but it now seems likely that it infected the plants through the soil, which was probably incompletely sterilized (the virus stands dry heat at 90°C. in soil without being completely inactivated).

For many years the tobacco necrosis viruses were thought of as laboratory curiosities, but in the late 1940's it was found that a serious disease, stipple streak, of beans (*Phaseolus vulgaris*) in Holland was caused by tobacco necrosis (Bawden and van der Want, 1949), and it was also found causing systemic infections of tulips (Kassanis, 1949), *Primula obconica* (Bawden and Kassanis, 1947), and a number of other plants. The viruses responsible are stable in the soil for long periods of time and are spread about by the commerce in such perennial plants as tulips and daffodils. The term "tobacco necrosis" was first given to this group of viruses because of the symptom picture in tobacco, but it is now realized that the disease is caused by a

FIG. 18. Electron micrograph of turnip yellow mosaic virus contrast-stained with phosphotungstate (magnification:  $\times 500,000$ ). Note suggestion of substructure and compare with Fig. 17. Electron micrograph by Dr. S. Brenner and Mr. R. W. Horne.

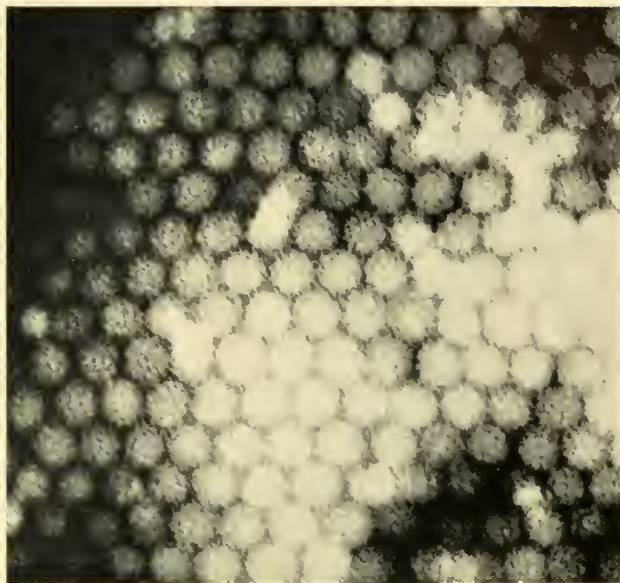


FIG. 19. Tobacco leaf inoculated with a tobacco necrosis virus. The concentration of virus used for the inoculation is suitable for local lesion counting, but is not sufficient for preparative purposes.

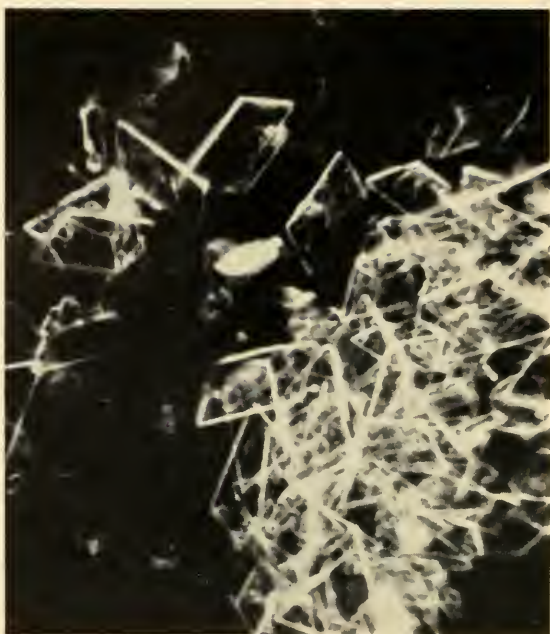


FIG. 20. Crystals of the common Cambridge strain of tobacco necrosis. Dark-ground illumination.



FIG. 21. Tobacco infected with a severe strain of potato X virus.

number of viruses which seem to have little in common except that they belong to the "spherical," small viruses and have similar host ranges. The group is very interesting but rather difficult to work with, because the quantity of virus obtained from infected plants is small, being of the order of 1 to 50 mg./liter of sap of tobacco leaves.

The tobacco necrosis viruses have a very wide host range, and are able to infect the roots of a large number of plants without causing much damage. It is only under exceptional climatic conditions that they infect the plants above the root region, but in that case they produce a very severe necrotic disease.

### *A. Purification*

The virus was first purified by Pirie and associates (1938), who took advantage of the stability of the virus to ethanol for clarification and precipitation (75 % ethanol was used, which is probably not suitable for all isolates of the virus) and this was followed by precipitation with ammonium sulfate. Finally the virus was crystallized in part as flat plates by the cautious addition of ammonium sulfate at 0°C. On standing at this temperature the virus crystallizes out (Fig. 20). The material was examined by the ultracentrifuge, and was found to contain several components. Chemical analysis showed that it was nucleoprotein in nature and contained some 14–18 % of nucleic acid of the ribose type. It also had the expected ultraviolet absorption of a nucleoprotein.

Some time later the problem was reinvestigated by Bawden and Pirie (1942), who multiplied up local lesion isolates obtained from various sources. These workers found that they were working with a group of different viruses, several of which could be traced to the earlier preparations because of the existence of antisera made to the latter. So undoubtedly the virus of Pirie and associates (1938) was a mixture. This is not generally found in plant virus work, where under such circumstances one strain will dominate the others after a while; but in this case the viruses in the mixture were unrelated, and as they caused only local lesions no interference between strains could take place.

Bawden and Pirie (1942) managed to characterize several of their isolates. They were named according to their origin, for example, Tobacco 1, 2, 3, and so on, Potato, Princeton, Rothamsted. The isolates Tobacco 1 and 2 are probably very similar, and crystallize as rhombic dodecahedra and also as bipyramids. Princeton, like many of the Cambridge isolates, crystallizes as flat plates, and most of these strains have sedimentation coefficients of about 115 *S*. (Price and Wyckoff, 1939) Rothamsted and the Dutch stipple streak are smaller and have  $s = 50$  *S*.



The chemical composition has not been investigated very intensively, but most of the isolates contain 1.7–2 % of phosphorus in the form of ribonucleic acid.

The Rothamsted strain apparently gives rise to two components (Bawden and Pirie, 1945a) and seen under the electron microscope spheres of 37 m $\mu$  and 17 m $\mu$  are present. The infectivity of the Rothamsted strain is associated with the larger particles, but the smaller particles are actively antigenic. It is tempting to suggest that the smaller particles were fragments of the protein of the larger forms and that the antigenic determinants were on the inside, but very little is known of the comparative chemistry of the two sorts of particle. That they are closely related is evidenced by the fact that both types of particles are produced by single-lesion isolates (Bawden and Pirie, 1950).

The plate-shaped crystals of tobacco necrosis isolates are exceedingly well-suited to observation by electron microscopy. Being so flat their surfaces are readily revealed by replica techniques (Markham *et al.*, 1948b) and this opens up a new method for the examination of virus crystals. The sensitivity of the method, however, is not to be compared even with that of the simplest X-ray diffraction methods, although the interpretation is more direct. For example, the crystal mentioned above gave the appearance of being cubic close-packed, with an interparticle spacing of 240 Å. The crystals were, however, quite appreciably birefringent when seen under polarized light, so that they could not belong to the cubic system (and were also evidently not hexagonal).

Crowfoot and Schmidt (1945) have investigated a crystal of a non-infectious derivative of the Rothamsted tobacco necrosis virus. This crystal was found in the ice chest and was particularly perfect, and large enough in size and lattice spacing to give many reflections without having to be oscillated. Unfortunately it was the only crystal of this material available, and was destroyed before the work was completed. A certain amount of confusion was caused by the authors trying to fit their observations to the results of the electron micrographs, which were from a quite unrelated specimen that crystallized in a different form.

It is quite evident that the tobacco necrosis viruses have not been studied as extensively as their interesting characteristics warrant. This is largely due to the difficulties of propagation and maintenance of stocks, but it is to be hoped that the work will be resumed using the more recent experimental techniques.

## XII. THE ALFALFA MOSAIC VIRUS

The alfalfa mosaic virus, an aphid-transmitted virus, was originally isolated by Ross (1941) from infected bean (*Phaseolus vulgaris*) plants by



high-speed centrifugation. The material obtained had 1.44 % of phosphorus, most of which was present as ribonucleic acid, and the sedimentation coefficient of the virus was usually small, being 74 *S*, the particles being spherical (Lauffer and Ross, 1940) or nearly so. The infectivity of the preparations was very low, and it now seems likely that the preparations were not mainly virus.

This virus has been investigated more recently by Bancroft and Kaesberg (1958), who followed the general methods of Ross, and found that the preparations contained three components with sedimentation coefficients of 73, 89, and 99 *S*, respectively. All contained ribonucleic acid and all were serologically similar. Only the 99 *S* component is infectious however. The other components look as if they might be fragments of the infectious component, which is oval in form, having dimensions of 20  $m\mu \times 55 m\mu$ . Only 14 % of the material is in the form of the 89-*S* substance while the other two are in equal amounts. The most rational explanation of the results of Ross and Lauffer is that their material was largely the 73-*S* component contaminated with a little 99-*S* component.

The alfalfa mosaic virus is unusual in that it is readily inactivated by digestion with trypsin, as is potato virus X.

### XIII. BROAD BEAN MOTTLE VIRUS

Bawden and associates (1951) have worked with a very unusual virus which affects *Vicia faba*, the broad bean. Up to 2 gm./liter of a nucleoprotein can be obtained from infected plants, although the sap is not very infectious itself. This is probably because the nucleoprotein itself has a very low infectivity, about  $10^{-4}$ – $10^{-5}$  gm./ml. being required for infecting plants. This is nearly a million times as much as is required for viruses such as tobacco mosaic, and the question arises whether the nucleoprotein is the virus. Certainly the healthy beans do not contain material of the type and the infective agent must have properties closely resembling those of the nucleoprotein. The latter is remarkable, too, in that it needs 75 % saturation with ammonium sulfate to cause the nucleoprotein to precipitate. This simplifies the purification a great deal, because it is only necessary to take the sap to half saturation with ammonium sulfate to clarify it, and then add an equal volume of saturated ammonium sulfate solution to precipitate the nucleoprotein. Purification is complicated by the large quantities of brown pigment produced in the bean sap.

#### A. Properties

The nucleoprotein consists of spherical particles of 17  $m\mu$  diameter as seen electron microscopically; they contain 1.6 % of P, at least 80 % of which is present as ribonucleic acid.

## XIV. THE SOUTHERN BEAN MOSAIC VIRUS

This virus received its name from its distribution in the southern states of the United States. Under natural conditions it causes a disease in *Phaseolus vulgaris* (Zaunreier and Harter, 1942), and it infects several leguminous plants, but its host range is fairly restricted, so that preparations are usually made on bean.

*A. Purification* (W. C. Price, 1946)

Bean plants are harvested 3 weeks after inoculation, frozen, and ground. Clarification is carried out using  $\text{Na}_2\text{HPO}_4$ , which may be used in solution to extract a second quantity of virus from the pulp. The virus is then precipitated by the addition of 300 gm. ammonium sulfate to each liter of sap, or it is centrifuged out [the sedimentation coefficient is 115 S: (Lauffer *et al.*, 1952)]. Further purification is carried out by the addition of ethanol to 30 % by volume, which causes extraneous material to precipitate. Removal of pigment from the preparations can be carried out by electrophoresis at pH 7. A Tiselius apparatus was used, but no doubt the more recent stabilized column electrophoresis apparatuses would be better for this purpose (Lauffer and Price, 1947).

Alternatively the virus can be adsorbed on to Amberlite XE-67, a basic resin, and eluted by  $\text{NaCl}$  + phosphate at pH 6.85, in which case the pigment is retained on the column. Trouble with pigment would also no doubt be bypassed by suitable treatment of the sap when preparing it.

The yield of virus is of the order of half a gram per liter of sap.

*B. Crystallization*

Crystals of the virus have been obtained in a number of ways. A 1 % solution of the virus in 20 % saturated  $(\text{NH}_4)_2\text{SO}_4$  is centrifuged, and the pellet free from the supernatant is moistened with water. The pellets become opaque on standing and contain masses of plate-shaped crystals, which are thought to be orthorhombic, but are not birefringent. The crystals are stable if stored in half-saturated  $(\text{NH}_4)_2\text{SO}_4$ . The virus also crystallizes out if dialyzed against tap water and pH 5.5 acetate buffer or against distilled water (Miller and Price, 1946a,b).

Crystals of this virus are of some interest because they were the first crystals to be examined by the electron microscope. One interesting point about this is that Wyckoff (1949) has published pictures of one of these crystals in which the packing distances in two planes roughly at right angles to each other are 1.5 : 1, suggesting that the virus particles themselves may have an axial ratio of this order (but see Labaw and Wyckoff, 1957). The

particles, as seen by themselves on electron microscopy, are essentially spherical or, more probably, polygonal (Kaesberg, 1956).

### *C. Physical Properties*

The virus, like many other spherical viruses, is hydrated in solution, having a diameter of 286 Å (Leonard *et al.*, 1953). The partial specific volume is 0.700 and the molecular weight is 6.6 millions (Lauffer *et al.*, 1952). The isoelectric point of the type strain and of a yellow mutant derived from it is pH 5.9 (MacDonald *et al.*, 1949).

The virus is stable over a fairly narrow pH range, this being from pH 4 to 8.

### *D. Chemical Composition*

The virus contains about 21 % of nucleic acid, which has been shown by Dorner and Knight (1953) to be ribonucleic acid. The base composition is: adenine, 1.03; guanine, 1.04; cytosine, 0.92; and uracil, 1.01, on a molar basis. This is probably the closest to unity in ratios yet found. As with tomato bushy stunt virus the nucleic acid is not released when the virus is denatured by boiling.

## XV. POTATO VIRUS X (POTATO LATENT MOSAIC VIRUS)

The potato X virus is one of the more important viruses in the world. It affects a large percentage of all potatoes grown, and even in its mildest form may cause 10 % or more reduction in the potato crop (K. M. Smith and Markham, 1945). The potential loss of food in the whole world, where about  $2.5 \times 10^8$  tons of potatoes are grown, is staggering. Allowing 10 % infection with this virus, which is almost certainly an underestimate, the loss is probably over 2 million tons of food every year. The amount of attention paid to this virus is hardly in keeping with its economic importance.

The potato X virus is of some interest because it was probably one of the first viruses to be recognized as being mainly protein in nature (Bawden and Pirie, 1936), and it is unusual in that it is readily digested by trypsin.

Although primarily a pathogen of the potato, in which it spreads by plant-to-plant contact, the potato X virus will grow readily on a number of plants, and for experimental work is usually grown on tobacco (Fig. 21). This transfer of hosts results in a gross change in the ability of the virus to infect potatoes, a change which might be accompanied by chemical differences. An even more dramatic change is shown on inoculation of the virus to *Cyphomandra betacea* (Matthews, 1949a), when the severity of the disease produced on transfer back to tobacco is greatly increased.

*A. Purification*

The sap of infected tobacco plants contains about 100 mg./ml. of the virus, and it may be clarified by the addition of  $K_2HPO_4$ , or better by heating to 55°C. The virus may then be precipitated by 0.25 saturation with ammonium sulfate (Bawden and Pirie, 1938a; Bawden and Kleczkowski, 1948) or by adjustment of the pH to 4.5 or just below. It may also be concentrated by high-speed centrifugation (Loring and Wyckoff, 1937; Loring, 1938). The sedimentation coefficient is about 124 *S*. (Laufer and Cartwright, 1952).

The virus analyzes for a nucleoprotein with about 5 % of nucleic acid (Bawden and Pirie, 1938a) but contains an excess of carbohydrate. The quantity of the latter is variable. Some 75 % of the ribonucleic acid associated with the virus may be prepared by the (somewhat drastic) method of Johnson and Harkins (1929) and the denatured protein still has 1.5–2.5 % of carbohydrate associated with it.

The virus is precipitated by 50 % (v./v.) ethanol and denatured by 85 % ethanol, and also by heating to 66°C., or by sodium dodecyl sulfate. It is precipitated from salt-free solution by pancreatic ribonuclease, which, however, does not digest the nucleic acid until the virus is denatured (Bawden and Kleczkowski, 1948). On the other hand, the virus is readily digested by trypsin, or, even more readily, by chymotrypsin. This interesting characteristic has not been exploited so far, but Knight (1955a) has investigated the action of carboxypeptidase, and has found that the major amino acid liberated is alanine (as in cucumber viruses 3 and 4), but a fairly large number of other amino acids are also released at the same time.

One of the more characteristic properties of this virus is its tendency to form jellies. The virus particles themselves are long flexuous threads, about 500 m $\mu$  in length (Bode and Paul, 1955) and are thinner than are tobacco mosaic virus rods. Being flexible, they entwine, forming ropelike structures, and, particularly if they are treated with salt solutions, they form these jellies. This characteristic is one which has, no doubt, prevented potato X virus from being as extensively studied as has the tobacco mosaic virus, but is not at all a disadvantage as far as routine chemical examination is concerned. The precipitated, aggregated virus may be rendered soluble to a large extent by the use of trypsin, with the loss of a fraction of its infectivity, and it would be fascinating to see this followed by electron microscopy and some of the other recent methods.

The nucleic acids of a number of strains have been analyzed (Markham, 1953a; Knight, 1954) but the proteins of this interesting group of viruses have received very little attention. This is all the more surprising, because the immunological relationships of this group of viruses have been studied by several workers, and on the whole the group is even more diverse than



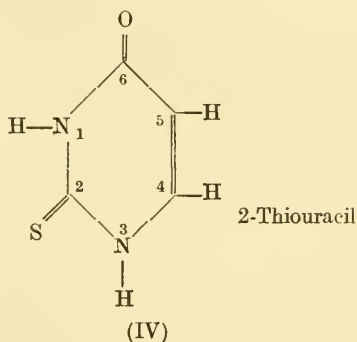
are the tobacco mosaic viruses (Spooner and Bawden, 1935; Matthews, 1949b).

Being elongated viruses, the potato X viruses show all the usual characteristics of such molecules. The solutions are viscous, show flow birefringence and form two layers on standing. The orientation of the liquid crystalline material is not as perfect as is that of the tobacco mosaic, and Bernal and Fankuchen (1941b) were unable to deduce much about the structure of the particles, except to find that they differed appreciably from the tobacco mosaics, and that the subunit size was not the same.

#### XVI. THE USE OF NUCLEIC ACID ANALOGS IN THE CONTROL OF PLANT VIRUSES

When it became evident that the nucleic acid of plant viruses played an important part in their economy, it was soon realized that the possibility existed in modifying the course of the disease by substances which might act as nucleic acid antagonists.

The earliest attempt of this kind was made by Commoner and Mercer (1952). It had been known for a long time that the tobacco mosaic virus contained uracil as part of the nucleic acid component, and Commoner and Mercer used the readily available analog 2-thiouracil, whose structure is shown in Formula IV,



in an attempt to inhibit nucleic acid synthesis and so to inhibit virus multiplication in tobacco. In fact thiouracil is exceedingly active against tobacco mosaic virus infection, but only at a concentration which is damaging to the plants. It has, however, been used to prevent spread of the virus in hypersensitive tobacco plants with some success (Holmes, 1955). The concentrations used are of the order of  $10^{-5}M$ , although higher concentrations have been used. Commoner and Mercer showed that the effect of thiouracil could be reversed by uracil. They prepared virus under conditions where inhibition was only partial, and they showed that the virus produced under



these circumstances was normal in its general properties. They also considered that the virus was as infective as the control virus grown on untreated tobacco. The system which they used to demonstrate this was unusually complex, but the general impression given by their data is that the virus grown in the presence of thiouracil is considerably less infectious than is the control material. Other workers have carried out more straightforward comparisons, and the results indicate that the thiouracil-treated virus is of similar infectivity to the control virus (Bawden and Kassanis, 1954).

Jeener and Rosseels (1953) made the important observation that the sulfur of the thiouracil was incorporated into the virus. They used  $S^{35}$ -labeled thiouracil for this purpose, and used tobacco leaves infected with tobacco mosaic virus floating on a liquid culture solution for growing the virus. From their counts of radioactivity they estimated that as much as 20 % of the uracil was replaced by thiouracil when the virus growth rate was reduced to half. The identification of the thiouracil was somewhat tentative, and the system which they used for the analysis actually decomposes thiouracil, which is rather unstable to the usual hydrolytic procedures.

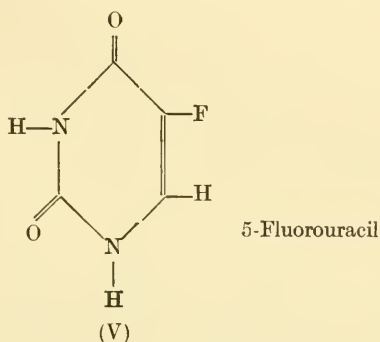
The fact that the thiouracil was actually incorporated into the virus as such was confirmed by Matthews (1956) after a number of unsuccessful attempts using whole plants. The amount of incorporation was about 3.5 % of the uracil or some 70 residues per virus particle. It would appear that little of the compound is incorporated unless the application is continuous, as it is with floating leaves. The inhibitory effect of the compound is shown in plants which have been sprayed, but under such conditions little or no thiouracil seems to be incorporated. That the compound is incorporated throughout the nucleic acid was shown by Mandel and associates (1957), who separated out the small polynucleotide fractions of treated virus.

Over half of the thiouracil was present in the ribonuclease digests as thiouridylic acid or as the adenine- and guanine-thiouracil dinucleotides. An interesting observation was that a quarter of the material was isolated as a substance identified by comparison with authentic thymidine 3' : 5'-diphosphate as thiouridine 3' : 5'-diphosphate, which should have much the same electrical charge distribution as the former. If this compound is not an artifact produced by enzyme contamination it would indicate that the thiouracil-containing virus would contain 10–20 breaks in the individual helices of nucleic acid. It is, however, evident that this work, like much of the structural work on virus nucleic acid, could bear repetition.

One very interesting observation has been recorded by Jeener (1957), who used virus containing 14–18 % of thiouracil substituting for uracil. He confirmed the earlier observations that this substitution had little or no effect on the infectivity of the virus. Equal concentrations of virus of both treated and control types were inoculated on to *Nicotiana glutinosa*; on 40

leaves the counts were 5392 against 5189 lesions, which is as good as equal. On the other hand, when tobacco leaves were inoculated with equal amounts of the same material, Jeener found that the multiplication rate was much less in the thiouracil-treated virus than it was in the control, the yield in his experiments being only about one-third. This strange phenomenon has not been investigated by other workers so far, but would really appear to deserve further examination. Jeener explains his results by assuming that the virus rod contains several infectious subunits, and that most of these can be inactivated by incorporation of thiouracil, but that one or a small number may survive, and then multiply at a reduced rate. This argument is not unequivocal, and the reduced rate of multiplication might, for example, be caused by an increased affinity of the virus for its protein coat, a possibility which might exist because of the increased acidic strength of the enolic groupings of thiouracil. Of course, as it is emphasized elsewhere, it is impossible to be absolutely certain about anything concerning viruses at the present time. It may even prove that the protein of thiouracil-treated virus differs from that of the untreated virus. This has not been investigated.

A compound allied to the last one is 5-fluorouracil, which differs from the natural base only in having a fluorine atom replacing a hydrogen atom (Heidelberger *et al.*, 1957). This substance, whose structure is shown in Formula V,

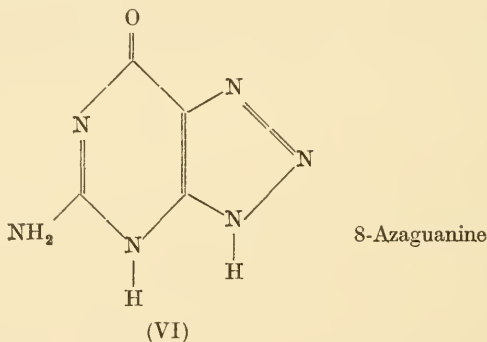


possesses a very low-grade inhibitory effectiveness for the virus, the concentrations needed being  $10^{-2}$ – $10^{-3}M$  (Davern and Bonner, 1958; Gordon and Staehelin, 1958). These concentrations are highly toxic to tobacco leaves. The quantity of fluorouracil incorporated is high, about 47 % of the uracil being replaced by its analog. The only other halogenated uracil likely to enter the ribonucleic acid of a virus is 5-chlorouracil, but this has not yet been investigated in such systems.

#### A. Azaguanine

Azaguanine is of interest as the first compound of an unnatural type to be isolated from nucleic acid (Matthews, 1953) in the form of nucleotides,

thus showing that the material was actually incorporated into the nucleic acid (the nucleoside 2'- and 3'-phosphates were isolated, showing that the nucleotide was originally present in diester linkage). Matthews had been working on the inhibition of virus multiplication which this compound caused. It is very inhibitory to the cucumber mosaic virus and to the alfalfa mosaic virus, and it has a lesser, but still marked, effect on other virus infections, including infection with the tobacco mosaic virus. Virus isolated from plants treated by spraying with the analog ( $10^{-2}M$ ) was hydrolyzed, using KOH, and the nucleotides liberated were separated by electrophoresis and by chromatography (Matthews, 1954). The separation is facilitated by two factors. First, azaguanine and its compounds fluoresce strongly and so may be detected in minute quantities; second, the dissociation constants of the groupings in the analog are changed, so as to make the compounds derived from azaguanine both more acidic and weaker bases than are the natural derivatives. The sensitivity of the detection procedure is such that  $0.3 \mu\text{g.}$  of azaguanine (Formula VI) may be detected in a mixture, so that the use of radioactive labels is unnecessary.



The similarity of structure between azaguanine and guanine is obvious, and, if anything, it is surprising that more of the compound is not incorporated into the virus nucleic acids. In the tobacco mosaic virus some 3-4 % of the guanine residues may be replaced, but this results in only a very minor reduction in the infectivity of the virus produced. In the turnip yellow mosaic virus even less of the unnatural compound is incorporated (Matthews, 1955) and the infectivity is reduced to about half that of the control virus.

One of the main deficiencies of azaguanine as an unnatural agent is its very short half-life. Almost all tissues of plants (and animals) contain an active guanase (guanine deaminase), which converts the compound to the inactive azaxanthine. This is probably one of the reasons why such high concentrations ( $10^{-2}M$ ) are needed for any appreciable biological effect to be manifested. However, in systems such as *Bacillus cereus* in which

there is no guanase activity, vast amounts of the unnatural analog may be incorporated into the ribonucleic acids before much biological change is evident, so that one must assume that azaguanine is relatively efficient in performing the function of guanine, at least in these bacteria.

One of the most remarkable features of the effect of purine analogs in virus infection is shown by tobacco mosaic virus-infected plants treated with 8-azaadenine. This compound is not incorporated into the virus nucleic acid in detectable amounts but is converted by the virus-host system into 8-azaguanine, which is found in the nucleic acid of the virus as the nucleotide (Smith and Matthews, 1957). The exact course of this remarkable transformation is uncertain. An even more surprising synthesis accomplished by the virus-infected plants is the production of 8-azaguanine from 4-(5)-amino 1-*H*-1 : 2 : 3-triazole-5-(4)-carboxamide, a synthesis which requires the formation of the pyrimidine ring by incorporation of a one-carbon fragment ( $C_2$  of the ring).

A number of other analogous and related compounds have been tried as antiviral agents, and the subject has been reviewed at length (Matthews and Smith, 1955). Most of the compounds investigated have very little effect or else the toxicity of the substances to the plants is too high for them to be used for a successful cure of virus disease.

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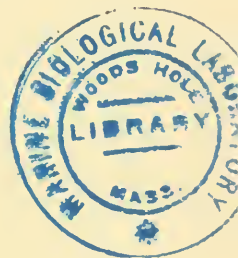
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## Chapter III

### Variation and Its Chemical Correlates

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#### I. INTRODUCTION

##### *A. Historical*

Evidence that viruses are not stable biological entities was provided by Pasteur's classic experiment with rabies at least a decade before it was

generally recognized that viruses were a distinct group of disease agents. Pasteur and his associates (1884) reported that intracerebral passage of the rabies agent through a series of rabbits resulted in a substantial decrease in the virulence of the rabies virus for its common host, the dog. Such a change persisted, that is, it was heritable, and the attenuated virus was designated "virus fixe" or "fixed virus." Since that time, mounting evidence has shown that variation is a very common characteristic of animal viruses. Indeed, this biological variability is both a blessing and a bane, for some of the most successful vaccines used to combat viral diseases of man and other animals have been made from attenuated viruses; while, on the other hand, the inevitable rise of new variants continually threatens the efficacy of these same vaccines.

Many years after Pasteur's experiments with rabies virus, Carsner and Stahl (1924, 1925) suggested that a similar attenuation of the virus of the curly-top disease of sugar beets was obtained by passage through nettle-leaved goosefoot, *Chenopodium murale*. Shortly afterwards, McKinney (1926) described the appearance of yellow spots on green mosaic-diseased tobacco plants. Transfers could be made from such spots to healthy plants where yellow mosaics were obtained rather than the green mosaics commonly associated with tobacco mosaic disease. These yellow mosaics were later suggested by McKinney (1929) to be variants of the mosaic virus and such a conclusion has been abundantly supported in the ensuing years.

These key observations on sugar beet curly top and tobacco mosaic viruses seem to have catalyzed a multitude of discoveries of plant virus strains, especially during the period 1930-1940. The investigations of Kunkel (1940) and Jensen (1933) revealed an astonishing number of mutants of tobacco mosaic virus (TMV), and similar findings, although usually on a smaller scale, were made over and over again with a wide variety of plant viruses (Kunkel, 1947). It seems safe to conclude now that few, if any, plant viruses do not mutate. In fact, the recognition that all viruses contain genetic material provides almost by definition that viruses should show mutations.

### *B. Terminology*

At this point it seems appropriate to discuss briefly terminology. Some workers speak of *variation* and others of *mutation* of viruses. Quite often the terms are used interchangeably. If mutation is considered to be a sudden random change in the hereditary material of an organism as a result of which the offspring bear a heritable characteristic different from the parents, it is clear that viruses mutate. There seems little basis at present for distinguishing between mutation and variation. The product of a heritable change in plant viruses is referred to as a *mutant* or *variant*, or very commonly as a

*strain*. The terms variant and strain are usually employed in a general sense while the term mutant is, in keeping with classical genetics, often reserved for those cases in which a highly specific relationship between parent and offspring is intended. For example, to say that the Holmes' ribgrass virus is a strain or variant of tobacco mosaic virus is simply stating that this virus belongs in the tobacco mosaic virus group. On the other hand, to say that the ribgrass virus is a mutant of tobacco mosaic virus would imply that it arose from tobacco mosaic virus by a specific mutational event involving a certain change in the genic constitution of the latter. There is no evidence that the ribgrass virus is such a mutant of TMV, whereas there is abundant evidence that it is a variant strain. Since in the general literature on viruses a rigorous distinction among terms is not always observed, the reader may be left to decide what is meant from the context. In the present treatment, the terms will be used in the sense defined above.

### *C. Frequency of Mutation*

Little effort has been made to determine quantitatively the frequency of mutation of plant viruses. This is largely attributable to the unfavorable sampling situation associated with the fact that thousands of virus particles must be applied to produce a single lesion for testing virus type. However, Kunkel (1940) found that when infectious juice from mosaic-diseased tobacco was rubbed on a leaf of a host which gave local lesions, about 1 lesion in 200 contained virus causing symptoms different from those of the original mosaic when returned to the systemic host. This result was obtained with virus inoculum obtained from plants about a month after the infected plants showed symptoms (this would be equivalent to about 5-6 weeks after inoculation); in a plant diseased for a short period of time the per cent of mutants was found to be smaller, while in plants diseased for several months as many as 1-2 % variants were observed. In any case, the mutation frequency, if representative of the total population, seems rather large when compared with that of the T-phages (Luria, 1953), certain mutations of which were found to occur with a frequency of 1 in  $10^3$  to 1 in  $10^9$  particles. It would be expected, of course, that different strains of TMV would have somewhat different mutation rates, and further, that other plant viruses might differ appreciably from TMV in this respect. Good quantitative comparisons are lacking but one gets the impression from the number of strains isolated (Kunkel, 1940) that common TMV mutates as frequently as or more frequently than most plant viruses. Despite this situation, the mass culturing of TMV and other plant viruses in appropriate plants results in a rather constant product consisting predominantly of progeny just like the strain inoculated. The inoculated strain apparently overgrows the mutant progeny,

although it is true that this relationship may be reversed by changing hosts.

It may be germane to mention here that the presence of about 0.5 % mutants in a plant virus preparation, while constituting a factor to be seriously considered in some types of investigation, can be neglected for most of the chemical and physical analyses.

#### *D. Significance of Mutation*

When a virus mutates, the resulting strain introduces new potentialities of disease. Virus diseases cause enormous damage to crop and ornamental plants, and whatever disease situation is prevalent, it cannot be considered stable. Mutation can occur to provide strains which are more or less invasive, more or less destructive, more or less readily transmitted by vectors, and more or less confined to a particular host plant. Furthermore, mutation may nullify the benefits of breeding "virus-resistant" varieties of plants by producing strains of virus to which the new hosts are not really resistant. As mentioned previously, it is true that a particular host provides a selective environment so that it is not dominated by one virus strain after another. Nevertheless, chance events do occur in nature which can favor the ascendancy of a mutant with all its potentialities.

Despite the considerable importance of the mutation phenomenon in the disease problems associated with plant viruses, mutation may be of even greater significance for its relevance to certain fundamental problems. One of these is the mechanism of virus multiplication. Since mutation is a discontinuous alteration of the normal process of virus reproduction, the study of strains may yield vital clues to the basic nature of virus reproduction itself. At least such studies should provide restrictions to which any comprehensive theory of virus duplication must conform.

Another area in which the study of plant virus mutation holds forth great promise is in the field of genetics. The plant viruses seem to be chemically and morphologically the simplest entities with genetic function which can be obtained in the quantities and in a state of purity suitable for extensive chemical and physical investigation. Viral strains are ideal here, for they possess different biological properties which it should be possible to relate to specific chemical structural differences. Thus, the virus mutants provide systems for a direct investigation of the chemical basis of heredity.

## II. PLANT VIRUS STRAINS

### *A. Characteristics of Strains*

Variants of plant viruses have been shown to differ in a variety of ways from the parent strains (Kunkel, 1947). With respect to disease, a strain may



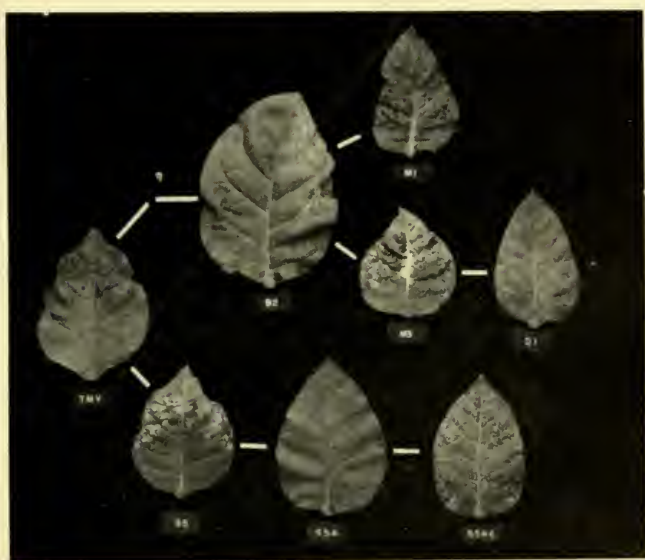
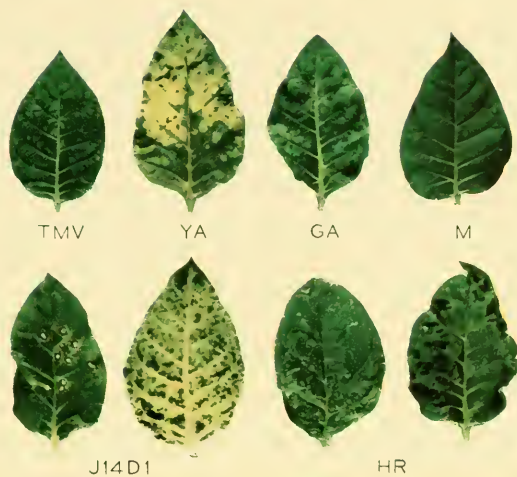


FIG. 1. Leaves from Turkish tobacco infected with different strains of tobacco mosaic virus. Top: TMV—tobacco mosaic; YA—yellow aucuba; GA—green aucuba; M—masked; J14D1—Jensen's derivative 1 from strain 14; HR—Holmes' ribgrass (Knight, 1947). Bottom: TMV and two serial groups of strains isolated by Drs. F. O. Holmes and M. Baylor, and F. L. Black (Black and Knight, 1953).



FIG. 2. Successive stages in the diseases caused in Turkish tobacco by TMV (top) and the strain J14D1 (bottom). As illustrated, plants infected with TMV will survive to the flowering stage and produce viable seed; in contrast, young tobacco plants infected with J14D1 strain are almost certainly killed, owing to the extensive necrotic effects of this strain.

cause different symptoms, a more or less severe disease, or different primary or secondary lesions. The variant may also differ from its parent strain in host range, in infectivity, in invasiveness, in specific insect vectors, and in mutability. The variant may have different thermal stability, serological properties, or chemical characteristics. However, in looking for mutants the first evidence sought is some indication of a change in the disease characteristics.

The wide variety of symptoms that can be caused by different variants of a plant virus in a given host is well exemplified by the strains of TMV. Some of these strains are so mild that in a given host no obvious symptoms are observed, and these variants are commonly called "masked" or "symptomless" strains. On the other hand, many strains cause stunting of growth and various kinds of distortion or malformation of the leaves; still others are distinguished by the different character or shades of their yellow or white mosaic patterns. Some strains cause spotting or striping of fruits borne on the infected plants, others do not. Some strains cause distinct necrotic effects, such as brown or white lesions, ringspot patterns, necrotic lines along the leaf veins, or stem necrosis. In short, the symptoms associated with strains of TMV can range from nothing discernible to complete annihilation by necrosis (Figs. 1 and 2).

### *B. Methods for Securing Strains*

Strains of a plant virus can be secured by a variety of methods. Some of the most useful will be listed and briefly described.

#### *1. Naturally Segregated Strains*

In any population of plants susceptible to virus disease and showing signs of infection there will be occasional plants with distinctly different symptoms. Thus, in a field of tobacco, it is possible to pick up new strains of tobacco mosaic virus by examining and selecting plants showing obviously different disease symptoms. Sometimes a population of plants may show a uniform disease pattern which by subsequent testing proves to be attributable to a virus not usually associated with that host. For example, there is a spinach blight caused by a strain of the common cucumber mosaic virus. Likewise, Holmes (1941) discovered an unusual strain of tobacco mosaic virus in a roadside weed, *Plantago*. The identification of a strange virus with a known one is accomplished by a series of tests to be described in the next section.

#### *2. Isolation from Distinctive Spots*

Bright yellow spots occasionally appear on leaves of plants infected with a virus that normally causes green mosaics or other symptoms (Fig. 3). Similarly, distinctive green areas are sometimes observed on leaves of plants

showing predominantly yellow symptoms. Somewhat less frequently, a necrotic brown lesion may appear on leaves showing mosaic patterns. Each of these distinctive spots represents an island of virus different from that in the major portion of the leaf, and if the virus is one transmissible mechanically, it can often be isolated by needle puncture of the spot, followed by puncture of a leaf on a healthy plant. There is evidence that similar transfers of new strains can be made by insects which by chance feed on a mutant spot and then on a normal plant (Hoggan, 1935).

An unusual instance of strain isolation from distinctive spots was reported by Best and Gallus (1955) working with strains of tomato spotted wilt virus. Mixed infection with two strains of this virus resulted in the production of pigmented necrotic lesions, from which a new strain was isolated having properties not coinciding with those of either of the strains used in the mixture causing infection.

### *3. Passage in a Different Host*

While virus strains are usually characterized by possession of a common host range, it is equally well known that a strain which does well in one host may do poorly in another, and the reverse. Hence, if a given strain is passed in a host in which it does not flourish, a new strain may gain the ascendancy. One passage, or, more often, several passages are required to secure a new strain by this technique. In any case, the origin of such a new strain is difficult to decide. A mutation may have occurred as a result of the altered environment to which the old strain was subjected, and the new strain may then have been selected by repeated passage in the new host whose environment favored it over the old strain. On the other hand, a mutant arising spontaneously in the first host may simply have been selected by passage in the new host. It is difficult to exclude the presence of such a mutant in the inoculum used on the second host, because the multiplication of mutants in any host is apt to be circumscribed owing to the interference phenomenon shown by related plant viruses (see Section II, C, 2). Hence, the mutant could constitute so low a percentage of the inoculum that it would never be detected save by some selective device, such as passage in a different host. The end result is, of course, the same, whether the new strain was already present in small amounts in the inoculum, or whether it arose by mutation of the first strain during multiplication in the second host.

### *4. Exposure to Elevated Temperatures*

Strains possess similar, although not identical, thermal stabilities. However, they multiply quite unequally at elevated temperatures. Hence, strains may be obtained by holding infected plant tissues or virus-containing insect



FIG. 3. A tobacco leaf showing symptoms of mosaic disease and displaying in the upper right portion a bright spot indicative of a variant strain of the mosaic virus.





vectors at higher-than-normal temperatures, but below inactivating temperatures. Here again the environment may be selecting a strain already present or it may be favoring certain mutations followed by selection.

### *5. Isolation from Local Lesions*

Several plant viruses, notably those transmitted mechanically, produce on certain hosts infections characterized by discrete local lesions. Each of these lesions appears to have arisen from a single infectious unit, since usually, although not infallibly, only a single strain can be recovered from such a lesion (Kunkel, 1934; Lauffer and Price, 1945; Benda, 1956). Hence, a method for securing new strains is to rub suitable dilutions of infectious juice on leaves of a host which gives the local response; individual lesions are cut out, ground in a mortar in a drop or two of buffer, and inoculated to one or more hosts suitable for distinguishing new strains.

### *C. Criteria of Strain Relationship*

Quite often, plant viruses are found in nature whose relationship, if any, to known viruses is obscure. The question then arises: is this a hitherto undescribed agent, or is it a strain of some previously reported virus? Likewise, when two or more distinguishable viruses are found in the same plant, the problem is whether these are strains of the same virus or unrelated viruses, for both situations appear to occur (Kunkel, 1944; Bawden, 1950; Bennett, 1953). Fortunately, two early observations provided a rational basis for an approach to these problems.

One of these was the finding that tobacco mosaic virus is antigenic and that closely related viruses (that is, viruses which on the basis of other properties are suspected of being strains) display serological cross-reactions (Purdy, 1928, 1929; Kunkel, 1947). The other significant observation was the demonstration of cross-protection from infection by closely related viruses (Thung, 1931; Kunkel, 1934). Thus, the first two criteria of strain relationship among plant viruses were immunological and biological in nature. These tests have been very useful over the years and are still widely employed. However, as studies on strains have progressed, additional criteria have developed. An attempt to collate the possible standards for strain relationship among plant viruses has been made (Knight, 1955a), yielding (with slight modification in the listing) the following criteria:

1. Positive serological cross reactions
2. Positive cross-protection tests
3. Similarity in host range
4. Similarity in method of transmission
5. Similarity in response to genetic change in host

6. Similar resistance to destruction of infectivity by chemical and physical agents, such as heat, desiccation, hydrogen ion concentration, irradiations, pressure, chemicals, etc.

7. Coincidence of specific chemical and physical properties:

- a. Particles same size and shape
- b. Same proportions of protein and nucleic acid
- c. Same proportions of nucleotides in nucleic acid components
- d. Same kind of *N*-terminal and *C*-terminal residues
- e. Same number of terminal residues and, hence, same number of sub-units in protein.

It should be noted that similar, though less extensive, standards were summarized by Findlay (1939). A brief explanation will now be given of each of the above criteria, together with selected references for more detailed information.

### 1. Serological Tests

Precipitin reactions, complement fixation, anaphylactic response, and neutralization of infectivity have all been used to test for the structural similarities which might denote strain relationship between plant viruses. The actual methods are well described in Bawden's book (1950).

The precipitin test has been most widely used, probably because of its relative simplicity and directness. If the viruses are closely related, this is indicated by copious precipitation when one of the viruses is mixed with antiserum for the other, and the reverse. If the relationship is not close, lesser amounts of precipitate will be observed up to the point of no precipitate and hence no serological relationship. One of the simplest applications of the precipitin test is illustrated in Table I with data obtained with some

TABLE I

PRECIPITATION OF SOME STRAINS OF TOBACCO MOSAIC VIRUS AND OF CUCUMBER VIRUSES 3 AND 4 WITH ANTISERUM AGAINST TOBACCO MOSAIC VIRUS<sup>a</sup>

| Antigen               | Dilution of antigen; 1 : 1 = 1 mg./ml. |   |   |       |   |   |        |   |   |              |   |   |
|-----------------------|--|---|---|-------|---|---|--------|---|---|--------------|---|---|
|                       | 1 : 1                                  |   |   | 1 : 4 |   |   | 1 : 16 |   |   | 1 : 64 1:256 |   |   |
| Tobacco mosaic (TMV)  | +                                      | + | + | +     | + | + | +      | + | + | +            | + | + |
| Yellow aucuba (YA)    | +                                      | + | + | +     | + | + | +      | + | + | +            | + | + |
| Green aucuba (GA)     | +                                      | + | + | +     | + | + | +      | + | + | +            | + | + |
| Holmes' masked (M)    | +                                      | + | + | +     | + | + | +      | + | + | +            | + | + |
| Holmes' ribgrass (HR) |  |   |   |       | + |   |        | + | + |              | + | — |
| J14D1                 | +                                      | + | + | +     | + | + | +      | + | + | +            | + | + |
| Cucumber 4 (CV4)      |  |   |   |       | + |   |        | + |   |              | + | — |
| Cucumber 3 (CV3)      |  |   |   |       | + |   |        | + |   |              | + | — |

<sup>a</sup> Degree of precipitation is indicated by the signs.

strains of tobacco mosaic virus and two cucumber viruses whose chemistry will be developed in later sections.

It is apparent from the data in Table I that all of the strains tested except HR (Holmes' ribgrass) are closely related serologically to TMV. That the antigens of two closely related strains, such as TMV and YA (yellow aucuba), are not identical can often be brought out by cross-absorption tests, as described by Bawden (1950). Another way of assessing the degree of relationship is by use of quantitative precipitin tests (Malkiel, 1947; 1948), although with certain strains of TMV this technique seems less able to reveal minor serological differences than cross-absorption tests combined with semi-quantitative precipitin reactions (Knight, 1942).

A major point about the serological tests is that viruses which are almost certainly strains by other criteria have thus far always shown positive serological cross-reactions. This fact has led some workers to conclude that the serological test is the most reliable evidence of strain relationship. In general, this supposition appears valid, although in the cases of weak serological cross-reactions it would seem to be assuming more than present knowledge of the chemical basis of serological specificity warrants. A case in point is the relationship of CV3 and CV4 (cucumber viruses) to the viruses of the tobacco mosaic group. While these cucumber viruses show an immunological relationship to the TMV group (Table I; and Roehow, 1956), they seem excluded on most other grounds (Knight, 1955a).

## 2. *Cross-Protection Tests*

Generally, a plant once infected with a virus remains infected throughout its life. Contact of the plant with other viruses capable of infecting that host usually results then in one of two major responses: (1) the plant is susceptible to infection by the second virus and new symptoms appear superimposed upon the first, or (2) the plant is resistant to infection by the second virus and the latter is excluded. When it can be shown that this exclusion is reciprocal, one has demonstrated cross-protection. (Synonyms include "mutual antagonism," "cross-immunization," and "interference.") Often protection can be shown conveniently in only one direction, and except for the most rigorous comparisons this may suffice.

Experience with plant viruses has shown, in general, that exclusion occurs between related viruses, that is, between mutants or other variant strains of the same virus group, whereas multiple infections occur with unrelated viruses. As Bennett (1953) has pointed out, there are exceptions to and modifications of the rule, depending somewhat on methodology, type of plant virus, and other circumstances. Despite these reservations the rule holds well enough to make it one of the most highly regarded criteria of strain

relationship. Furthermore, it gains strength when coupled with the serological tests, since there seems to be only one case reported (Bawden and Kassanis, 1945) in which serologically unrelated plant viruses showed strong cross-protection.

### 3. *Similarity in Host Range*

Plant viruses, in common with viruses in general, show a specificity for hosts in whose cells they can multiply. This specificity is narrow or broad, depending upon the plant virus (Holmes, 1938; Price, 1940). However, members of the same group of viruses would be expected to have similar requirements for host conditions suitable for their multiplication. While extensive systematic studies have not been made to test this assumption, observations in general attest to its validity. Hence, strains of a virus have similar host ranges, although, as pointed out earlier, they may not be identical. Obviously, this test for strain relationship is less highly specific than the serological or cross-protection tests; nevertheless, it can be quite significant.

### 4. *Similarity in Method of Transmission*

Some plant viruses are transmitted mechanically (such as by rubbing juice from an infected plant on the leaf of a healthy plant), others are transmitted by grafting, by means of parasitic plants, or by insect vectors. Some viruses may be passed by several or all of these means. In the case of insect transmission, specificity is often shown by the type of insect vector and by whether or not the virus undergoes a latent period in the vector and persists for more than a limited time. Members of the same virus group are commonly transmitted by the same methods, but sometimes unrelated viruses are, too (Bawden, 1950). Thus, similarity in method of transmission can help to show strain relationship between viruses, although this would be considered insufficient if it were the sole evidence for strain relationship.

### 5. *Similarity of Response to Genetic Change in Host*

This test has not been widely used, largely because of the restrictions imposed by the test plants required. In brief, one must have two groups of test plants differing specifically in genetic constitution, which in turn must result in distinguishably different disease symptoms when a given virus is inoculated to plants of the two sets. It is then found that viruses which belong to the same virus group as the first one (that is, are strains) likewise cause, when inoculated to the two sets of plants, two sharply distinguishable disease types. Viruses unrelated to the one for which the test plants were established, but which can infect these hosts, produce indistinguishable diseases in the two sets of plants.



This test was used nicely by Holmes (1941) to help demonstrate that a *Plantago* virus was actually a strain of tobacco mosaic virus.

#### 6. *Similar Resistance to Destruction of Infectivity*

The earliest studies on the properties of plant viruses (Stanley, 1938; Smith, 1939, 1957; Bawden, 1950) showed considerable disparity in the resistance of different viruses to chemical and physical agents. However, viruses of the same group show remarkably similar, though often not identical, ability to survive treatment with various chemical and physical agents. This, then, becomes another standard by which strain relationship or lack thereof can be judged. This criterion, like those of similarity of method of transmission and similarity of host range, is less specific than the immunological standards, but when pooled with other evidence can be of considerable weight.

Some of the treatments to which viruses might respond in characteristic manner include subjection to heat, pressure, irradiation, desiccation, and chemicals (especially salts of heavy metals, oxidizing agents, formaldehyde, enzymes, acids, and bases).

#### 7. *Coincidence of Specific Chemical and Physical Properties*

Plant viruses possess a variety of sizes and shapes, including forms which appear in electron micrographs as stiff or sinuous rods, spheroids, and particles with hexagonal contour (Williams, 1954). However, individual plant viruses or strains of a virus are characterized by particles which are so similar in size and shape that they are not distinguished at present by behavior in the analytical ultracentrifuge, X-ray scattering, or electron microscopy (Stanley, 1943; Bernal and Fankuchen, 1937, 1941; Knight and Oster, 1947; Steere, 1953). It is possible, of course, for two unrelated viruses to have particles of the same size and shape, although very few instances of this kind are known at present; therefore, with this reservation in mind, a coincidence of particle size and shape for two viruses is a good piece of evidence for strain relationship.

No highly purified plant virus appears at present to contain constituents other than nucleic acid and protein. Just as different viruses vary in size and shape, so also do they differ in their proportions of nucleic acid and protein. The viruses of the tobacco mosaic group contain only about 5 % nucleic acid, whereas turnip yellow mosaic and tobacco ringspot viruses contain about 35 %; other viruses contain intermediate amounts. The significant point is that virus strains appear to contain the same proportions of nucleic acid and protein, and hence analyses for these constituents can help to characterize a virus.

Studies of the chemical compositions and structures of strains of TMV have shown that strains possess highly characteristic common features, such as nucleic acids of the same composition, peptide chains terminating with the same amino acid residues, and macromolecular structures possessing the same numbers of subunits. These features will be elaborated in the sections devoted to the protein and nucleic acid components of strains.

#### *D. Evaluation of Criteria of Strain Relationship*

From the point of view of establishing strain relationship, applicability and specificity of the tests employed are both of prime importance, but, fundamentally, specificity is clearly paramount. What then are the most specific criteria? As will be described in later sections, mutation of plant viruses seems, on the basis of present information, to result in relatively small changes in the major chemical and physical properties of viruses. Even those variants which differ most widely in biological properties have been found to possess certain apparently identical physical and chemical characteristics not shared with unrelated viruses. Therefore, the coincidence of specific major chemical and physical properties would seem to provide the best evidence for strain relationship. Since the immunological reactions are doubtless based on chemical structure, it seems appropriate that immunological tests be regarded second only in specificity to the structural characteristics themselves. By comparison, all of the other criteria enumerated may be considered inferior to the physicochemical and immunological standards with respect to specificity, with the possible exception of Holmes' test for response to a genic change in the host.

Turning to applicability, it can be pointed out that relatively few of the 300 or so plant viruses presently known (Smith, 1957) have been obtained in a state permitting precise physicochemical characterization. On the other hand, immunological tests can often (though not always) be successfully performed on small amounts of crude materials. In proper perspective, then, the physicochemical tests for strain relationship are to be preferred for the ultimate in specificity, but from the point of view of practicality, the one grade less specific but widely applicable immunological tests may be for the present more generally useful.

Another pertinent question is how many of the criteria of strain relationship must be satisfied in order to establish that two plant viruses are variant strains belonging in the same group of viruses. It seems to the writer that this question is answered variably by different workers, but that the following principles may be suggested: (1) In general, greatest reliance should be placed on the outcome of the most specific tests, namely, the physicochemical and immunological tests. (2) The more criteria satisfied, the stronger is the

case for strain relationship. It will be recognized, of course, that none of the tests is infallible. All of them have limitations and these limitations need especially to be considered in borderline cases.

### III. CHEMISTRY OF VIRUS STRAINS

The plant viruses isolated and purified thus far have proved to be solely nucleoproteins. The chemistry of these viruses is therefore conveniently resolved into studies of protein and nucleic acid components.

#### *A. Protein Components*

##### *1. Composition of Strain Proteins*

The common amino acids found in most proteins are also found in viruses. No unusual amino acids, D-isomers, or extraordinary amounts of individual amino acids have been observed so far. Thus, there is nothing unique about viral protein compositions which clearly distinguishes them from other biologically active agents. However, individual viruses, and often strains of the same virus, have been found to possess distinct and characteristic protein components.

The tools for complete analysis of proteins for their amino acid content have been available since only about 1945. First, microbiological assay and then ion exchange chromatography have been applied to the products of acid and alkaline hydrolysis of the viruses. The agreement of results obtained by the two methods has been surprisingly good in the few instances in which appropriate comparisons have been made.

In Table II are given the results of some amino acid analyses of a few plant viruses. These illustrate the differences between unrelated viruses and provide a basis for comparison with the results obtained with different strains. The values reported for TMV by Ramachandran (1958) and shown in column 2 are probably the most accurate now available for this widely studied virus.

*a. Strains of Tobacco Mosaic Virus.* The variant strains of TMV have been more widely studied than strains of any other virus for several reasons. Historically these were among the first to be recognized as variants of a plant virus; subsequent studies have shown them to be very numerous [over 400 reported (Kunkel, 1947)], generally easy to isolate and to purify, readily transmitted, and characterized by distinctive qualitative and quantitative symptoms in appropriate easily grown hosts.

Physical-chemical studies have been made on various strains of TMV in laboratories in different parts of the world. However, certain selected strains familiar to the author will be used primarily to develop the picture of strain chemistry, not because these strains are more important than others, but

because the most extensive and best integrated studies have been made on them. These strains are illustrated in Fig. 1, which shows some of the symptoms that they cause in one host, Turkish tobacco. The strains shown at the

TABLE II  
AMINO ACID CONTENT OF SOME PLANT VIRUS PREPARATIONS <sup>a</sup>

| Constituent                            | Virus <sup>a</sup> |                  |      |                  |                | References  |
|--|--------------------|------------------|------|------------------|----------------|---|
|  | TMV <sup>a</sup>   | TMV <sup>b</sup> | CV4  | BSV              | TYMV           |   |
| (Gm. amino acid residue/100 gm. virus) |                    |                  |      |                  |                |   |
| Alanine                                | 4.1                | 5.6              | 4.9  | 4.7              | 2.8            | For TMV and<br>CV4, Knight (1947)<br>and adapted from<br>Ramachandran<br>(1958); for BSV, de<br>Fremery and<br>Knight (1955); and<br>for TYMV, Fraser<br>and Cosentino<br>(1957). |
| Arginine                               | 8.8                | 8.9              | 8.3  | 5.7              | 1.3            |   |
| Aspartic acid                          | 11.7               | 11.6             | 11.3 | 9.2              | 3.6            |   |
| Cysteine                               | 0.6                | 0.5              | 0.0  | 0.6 <sup>c</sup> | — <sup>d</sup> |   |
| Cystine                                | 0.0                | — <sup>d</sup>   | 0.0  | — <sup>d</sup>   | 0.0            |   |
| Glutamic acid                          | 9.9                | 11.2             | 5.7  | 5.0              | 4.6            |   |
| Glycine <sup>e</sup>                   | 1.4                | 1.8              | 1.1  | 4.0              | 1.9            |   |
| Histidine                              | 0.0                | —                | 0.0  | 1.2              | 0.9            |   |
| Isoleucine                             | 5.7                | 5.3              | 4.0  | 2.7              | 4.2            |   |
| Leucine                                | 8.0                | 7.7              | 8.1  | 8.9              | 4.8            |   |
| Lysine                                 | 1.3                | 1.35             | 2.1  | 3.1              | 2.9            |   |
| Methionine                             | 0.0                | — <sup>d</sup>   | 0.0  | 0.7              | 1.2            |   |
| Phenylalanine                          | 7.5                | 6.2              | 8.7  | 3.7              | 2.1            |   |
| Proline                                | 4.9                | 4.0              | 4.8  | 2.8              | 6.4            |   |
| Serine                                 | 6.0                | 8.3              | 7.8  | 5.6              | 3.6            |   |
| Threonine                              | 8.4                | 9.0              | 5.9  | 8.1              | 7.4            |   |
| Tryptophan                             | 1.9                | 2.4              | 0.5  | 0.6              | — <sup>d</sup> |   |
| Tyrosine                               | 3.4                | 3.3              | 3.3  | 3.1              | 0.8            |   |
| Valine                                 | 7.8                | 7.3              | 7.5  | 7.3              | 3.4            |   |
| Total                                  | 91.4               | 94.45            | 84.0 | 77.0             | 51.9           |   |
| % Nucleic acid                         | 5                  | 5                | 5    | 17               | 35             |   |

<sup>a</sup> The following abbreviations are used: TMV, tobacco mosaic virus; CV4, cucumber virus 4; BSV, bushy stunt virus; TYMV, turnip yellow mosaic virus.

<sup>b</sup> TMV values from microbiological assay.

<sup>b</sup> TMV values from ion exchange chromatography.

<sup>c</sup> Cysteine was determined in the native virus by the method of Tsao and Bailey (1953).

<sup>d</sup> Dash indicates that no analysis was made for the amino acid indicated.

<sup>e</sup> A correction was made in the case of BSV for glycine derived from nucleic acid degradation, but not in the other cases.

top of Fig. 1 were selected from available ones at the Rockefeller Institute for their distinctive biological properties. Some are closely related to ordinary TMV and others not (Knight, 1947). The strains illustrated at the bottom of



Fig. 1 were obtained by procedures thought to yield direct series of mutants (Black and Knight, 1953). The differences in biological properties of these strains are not limited to the quality of the symptoms they elicit, for some show striking differences in the severity of the disease they cause. This is illustrated in Fig. 2, which contrasts the course of infection with ordinary TMV with that resulting from the strain J14D1. Purified preparations of these biologically diverse strains constitute the materials for chemical investigation.

The primary operation in protein chemistry is to determine the kinds and amounts of amino acids present. Such analyses are important, not only for the light they shed on the nature of the protein concerned, but also because such information is basic for subsequent structural studies.

The first complete amino acid analyses on viruses were made on the 6 distinctive strains of TMV that cause the symptoms illustrated in the top half of Fig. 1 (Knight, 1947). Later, 7 other strains, whose symptoms are shown in the bottom half of Fig. 1, were also analyzed (Black and Knight, 1953). Microbiological assays were used except for cysteine, and, in one series, arginine and tryptophan, for which colorimetric methods were employed. The results of the analyses made on the above 13 strains of TMV are summarized in Table III, in which values for the strains are given only in the cases that are considered to differ significantly from TMV. The values shown were obtained by many analyses on different preparations of the strains. An arbitrary level of significance of 10 % was chosen; in general, no strain was considered to have a significantly different amino acid content unless it differed from TMV by this much or more. Such a margin of error is considerably greater than the mean deviation of single analyses from the average and hence the estimate of significant differences is conservative. Supporting evidence for the validity of this evaluation has come from subsequent studies in which isoelectric points, peptide analysis, and other characteristics of the preparations have proved consistent with the results reported for the amino acid analyses (Oster, 1951; Knight, 1957). However, a difficulty was encountered with the microbiological assay in that in an interval of 5 years between two major groups of analyses, a shift in level of values for some of the amino acids was observed. This was due to changes in the microorganism used for assay rather than in the preparations, since the same answer was obtained with all preparations from the current ones to those 10–15 years old. For this reason, two sets of values are given for TMV in Table III, one for each of the two major periods of analysis. From these data, it can be concluded that some strains of TMV do and some do not differ demonstrably in protein composition.

*b. Strains of Tomato Bushy Stunt Virus.* For many years bushy stunt virus (BSV) was thought to be an exception to the rule that plant viruses mutate.



However, Steere (1953) was able to demonstrate distinctive strains of this virus by inoculating the juice from systematically infected plants to a local

TABLE III  
DIFFERENCES IN AMINO ACID CONTENT BETWEEN TOBACCO MOSAIC VIRUS AND SOME OF ITS STRAINS

| Amino acid    | TMV            |      | Virus Strain |       |      |      |      |      |                                      |      |      |     |     |     |  |  |
|---------------|----------------|------|--------------|-------|------|------|------|------|--------------------------------------|------|------|-----|-----|-----|--|--|
|               |                |      | M            | J14D1 | GA   | YA   | HR   | B2   | B2A                                  | B3   | B4   | S1  | S2  | S3  |  |  |
|               | 1 <sup>a</sup> | 2    |              |       |      |      |      |      |                                      |      |      |     |     |     |  |  |
|               |                |      |              |       |      |      |      |      | gm. amino acid residue/100 gm. virus |      |      |     |     |     |  |  |
| Alanine       | 4.1            | 5.9  | —            | —     | —    | —    | 5.1  | —    | 7.7                                  | 7.8  | —    | 4.7 | 4.5 | 5.3 |  |  |
| Arginine      | 8.8            | 8.7  | —            | —     | 10.0 | 10.0 | —    | —    | 9.9                                  | —    | —    | —   | —   | —   |  |  |
| Aspartic acid | 11.7           | 10.4 | —            | —     | —    | —    | 10.9 | 12.3 | —                                    | —    | 12.7 | —   | —   | —   |  |  |
| Cysteine      | 0.6            | 0.6  | —            | —     | —    | —    | —    | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Glutamic acid | 9.9            | 9.7  | —            | 9.1   | —    | —    | 13.6 | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Glycine       | 1.4            | 1.9  | —            | —     | —    | —    | 1.0  | —    | 2.5                                  | —    | —    | —   | —   | —   |  |  |
| Histidine     | 0.0            | 0.0  | —            | —     | —    | —    | 0.6  | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Isoleucine    | 5.7            | 5.1  | —            | —     | 4.9  | 4.9  | 5.1  | —    | —                                    | —    | —    | 6.3 | 6.0 | 6.2 |  |  |
| Leucine       | 8.0            | 6.9  | —            | —     | —    | —    | —    | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Lysine        | 1.3            | 1.2  | —            | 1.7   | —    | —    | —    | —    | 1.9                                  | —    | —    | —   | —   | —   |  |  |
| Methionine    | 0.0            | 0.0  | —            | —     | —    | —    | 1.9  | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Phenylalanine | 7.5            | 7.3  | —            | —     | —    | —    | 4.8  | —    | —                                    | —    | —    | 6.1 | 5.9 | 6.1 |  |  |
| Proline       | 4.9            | 4.6  | —            | —     | —    | —    | —    | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Serine        | 6.0            | 7.6  | —            | —     | —    | —    | 4.7  | 8.8  | —                                    | 8.8  | 9.1  | —   | 8.7 | 9.0 |  |  |
| Threonine     | 8.1            | 10.1 | —            | —     | —    | —    | 7.0  | 8.4  | —                                    | 11.0 | 12.1 | —   | —   | —   |  |  |
| Tryptophan    | 1.9            | 1.7  | —            | —     | —    | —    | 1.3  | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Tyrosine      | 3.4            | 3.3  | —            | —     | —    | —    | 6.1  | —    | —                                    | —    | —    | —   | —   | —   |  |  |
| Valine        | 7.8            | 9.3  | —            | —     | —    | —    | 5.3  | —    | 9.9                                  | 10.3 | 10.8 | —   | —   | —   |  |  |

<sup>a</sup> The first set of values for TMV is the one against which the strains over to HR should be compared. From B2 through S3 the values should be referred to the second set of values for TMV. See text. For ease of comparison, only those values are given for the strains that are considered to differ significantly from the figures for TMV. The data were calculated from Knight (1947), and from Black and Knight (1953).

lesion host and then transferring from individual local lesions back to systemic host plants. Three strains showing distinctive symptoms in *Datura stramonium* plants are illustrated in Fig. 4. These strains were designated



FIG. 4. Plants of *Datura stramonium* infected with three strains of tomato bushy stunt virus. From left to right: BS3, BS9, BS10 (de Fremery and Knight, 1955).



BS3, BS9, and BS10, and their protein components were analyzed by the Moore and Stein technique of ion exchange column chromatography supplemented by colorimetric procedures (de Fremery and Knight, 1955). No decisive differences were found and it was concluded that the amino acid compositions of the three strains are very similar, if not identical. A composition which could pass for all three of the strains is given in Table II.

From the above, it can be concluded that the situation with respect to the protein compositions of these particular BSV strains is like that of only the most closely related TMV strains in which also no differences in amino acid content were observed.

*c. Summary of Compositions of Strain Proteins.* From data of the sort indicated in Table III certain conclusions can be drawn:

1. Strains often differ from one another in protein composition. These differences usually are manifested by different proportions of constituent amino acids. In some cases amino acids may be present in one strain and entirely lacking in another. The HR strain exemplifies this situation (see Table III).

2. In general, there is little evidence for pronounced change in protein composition during individual mutational steps, for the closely related strains are found to be most similar in composition. In fact, in some cases, as with TMV and M, and with three strains of BSV, it has not been possible as yet to distinguish between the proteins on the basis of composition.

3. Differences in strain proteins seem to involve almost any of the various amino acids. Only three, cysteine, leucine, and proline, appear thus far to be constant in the thirteen strains of TMV analyzed.

## *2. Structural Features of Strain Proteins*

A logical extension of the compositional studies on virus strains is the investigation of structural features. Of course, the size and shape of virus particles are based on their chemical structure, but thus far no significant differences in morphology between virus strains have been demonstrated. One can, therefore, tentatively conclude that the gross structures of the strains are very similar, if not identical. On the molecular level, however, there are many possibilities for differences between strains. In the case of the protein, there are the questions of how many peptide chains there are, how they terminate, how they are arranged in space, and how they are linked to the nucleic acid. Within peptide chains, there is the question of sequence of amino acid residues. These and similar problems are under investigation and some of the results obtained will be described.

*a. Some Structural Features of TMV and Strains.* Early studies of the low-angle scattering of X-rays by TMV and some of its strains (Bernal and Fankuchen, 1941) revealed a regularity of structure so great that it was

suggested that the individual particles of TMV resemble crystals and that they are composed of subunits. Subsequent studies by the X-ray technique have supported this idea (Franklin, 1957). Further suggestion that TMV particles might possess a substructure was provided by the observations of Schramm and colleagues (Schramm, 1954) that TMV seemed to degrade in a regular, rather than a random manner, and that certain degradation products could be repolymerized to give back virus-like particles. Also, Takahashi and Ishii (1952, 1953) discovered a low molecular weight protein in mosaic infected tobacco (so-called X-protein) which could be polymerized to give virus-like rods. Compelling chemical evidence for the presence of subunits in TMV structure was provided when it was found (Harris and Knight, 1952, 1955) that carboxypeptidase caused the release of about 2900 threonine residues from each mole of TMV (assuming a molecular weight of  $50 \times 10^6$  for TMV). These carboxyterminal (*C*-terminal) threonine groups are considered to represent an equal number of peptide chains in the virus structure. Hence, the virus is composed of subunits, each with a molecular weight of about 17,000, or, subtracting the nucleic acid, a protein subunit of about 16,000.

The thirteen different strains of TMV illustrated in Fig. 2 were also treated with carboxypeptidase, and it was found that they all had *C*-terminal threonine, and in approximately the same amount (Knight, 1955b). Hence, it can be concluded that these strains of TMV probably contain the same numbers of subunits in their structures and that the peptide chains terminate in the same way. Since unrelated viruses gave results which were qualitatively and quantitatively different, the identity of *C*-terminal groups seems to be a chemical criterion of strain relationship.

Further information concerning the *C*-terminal end of the peptide chains of the strains was obtained by a study of the peptides released from strain proteins by chymotrypsin (Niu and Fraenkel-Conrat, 1955). *C*-terminal hexapeptides were obtained from the strains TMV, M, YA, and HR, and these proved to have identical amino acid sequences in the cases of TMV, M, and YA, while the hexapeptide from HR had the same sequence for the last three residues, but differed in the other three.

There appears to be no free amino terminal (*N*-terminal) end of the peptide chains of TMV (Fraenkel-Conrat and Singer, 1954). Recently, Narita (1958) isolated an *N*-acetylated peptide containing serine and tyrosine from enzymatic digests of TMV protein and this is thought to represent the *N*-terminal structure of the virus protein. This same peptide has also been obtained from the strains M, YA, HR, and J14D1 and hence is probably a characteristic feature of strains of TMV (Narita, 1958b).

The following sketch appears now to represent the terminal features of TMV strains:

*N*-acetylseryltyrosyl . . . prolylalanylthreonine



In between the two ends of each subunit peptide chain there are in the neighbourhood of 160 amino acid residues. To determine the sequence of these is a formidable task. However, methods are available which permit a rather rapid survey of the chain by splitting it into a series of peptide fragments. For example, treatment of the TMV protein with trypsin yields about 13 peptides, which can be separated by a two-dimensional paper electrophoresis-paper chromatography procedure (Knight, 1957). By treatment of the paper with a suitable reagent that develops a color by reaction with the peptides, a characteristic map of the peptides is obtained. This technique has been applied to only a few strains of TMV, with the following results: The peptides of TMV and M appear to be so similar that it has not been possible as yet to distinguish between their maps. On the other hand, the peptide maps of TMV and HR are widely different, as might be anticipated from the very different amino acid compositions of the two strains. The peptides of YA appear to coincide in main with those of TMV, but differ in three or four instances. Isolation of quantities of peptides by such procedures as countercurrent distribution and ion exchange column chromatography is making possible the determination of amino acid sequences of individual peptides; eventually these will be put together to develop complete sequences for the various strain proteins.

*b. Structural Features of Plant Viruses Not in the TMV Group.* Little has been done as yet on the structures of other plant virus strains. However, bushy stunt virus, potato virus X, and cucumber viruses 3 and 4 have been found, like TMV, not to have free *N*-terminal residues while having characteristic kinds and numbers of *C*-terminal groups (Niu *et al.*, 1958). The absence of free *N*-terminal groups in these viruses appears to be explained, as in the case of TMV, by the presence of *N*-acetyl, but each virus has been found to have a different and characteristic acetyl-*N*-terminal amino acid sequence (Narita, personal communication).

*c. Summary of Structural Features of the Proteins of Virus Strains.* There are as yet too few data to generalize extensively on the essential structural features of plant virus strains. Nevertheless, the picture seems so clear with the TMV strains that the following theory can be proposed for further testing as new information becomes available. It is assumed that the protein moieties of all macromolecular plant viruses are made up of subunits (Crick and Watson, 1957). There is evidence to support this assumption in every case so far examined (TMV, BSV, PXV, CV3, and CV4). Each variant strain of a group of viruses will have the same number of subunits, which will be indicated by the presence of equal numbers of terminal amino acid residues. These terminal residues will not only be equal in number but also in kind.

*B. Nucleic Acids*

All plant viruses so far examined have been found to contain ribonucleic acid (RNA). As shown in Table IV, the amount of RNA in different viruses

TABLE IV  
APPROXIMATE QUANTITY OF RIBONUCLEIC ACID IN SOME  
PURIFIED PLANT VIRUS PREPARATIONS

| Virus                          | Per cent. RNA  | References   |
|--------------------------------|----------------|--|
| Alfalfa mosaic                 | 15             | Ross (1941)  |
| Broad bean mottle              | 18             | Bawden <i>et al.</i> (1951)                          |
| Potato X (latent mosaic)       | 6              | Bawden and Pirie (1938)                              |
| Southern bean mosaic           | 21             | Miller and Price (1946)                              |
| TMV and strains                | 6 <sup>a</sup> | Knight and Stanley (1941)<br>Black and Knight (1953) |
| Tobacco necrosis               | 18             | Bawden (1950)  |
| Tobacco ringspot               | 34             | Steere (1956)  |
| Tomato bushy stunt and strains | 17             | de Fremery and Knight (1955)                         |
| Turnip yellow mosaic           | 35             | Markham (1953)                                       |

<sup>a</sup> Recent evidence indicates that the precise value is probably closer to 5 % than to 6 (Knight and Woody, 1958).

ranges from about 5-6 % for TMV and potato virus X to about 35 % for tobacco ringspot and turnip yellow mosaic viruses. Whatever the amount of RNA, it seems to be characteristic for a group of viruses, the variant strains; at least, with many strains of TMV and three of BSV it has not been possible to detect significant differences in nucleic acid content among strains of the same virus. The proportion of RNA in a virus thus becomes a chemical criterion of strain relationship, with the reservation that alone this is insufficient evidence in view of the fact that some unrelated viruses have been observed to have comparable amounts of RNA.

### 1. *Compositions of Strain Nucleic Acids*

*a. Strains of TMV and of Bushy Stunt Virus.* Analyses of nucleic acids for constituents are usually preceded by application of acid or alkaline hydrolytic procedures. The best results can be expected when the procedures are applied to isolated nucleic acids; however, the analytical methods have been successfully used on whole viruses and the protein components seem to cause little or no difficulty, especially in those cases where the acid is 10 % nucleic or more. The methods for analyzing nucleic acids are well described by Markham and Smith (1954) and in Chargaff and Davidson's monograph (1955).

The three major constituents of nucleic acids are phosphoric acid, a pentose sugar, and the bases—i.e., purines and pyrimidines. The first two are not particularly distinctive, although it could be quite significant were the pentose other than ribose. Ribose has been positively identified as the sugar in TMV and some of its strains (Knight, 1954), and it has been shown by chromatographic methods to be the purine-bound sugar in strains of BSV (de Fremery and Knight, 1955) and in turnip yellow mosaic virus (Markham and Smith, 1951). However, in most plant viruses, the presence of ribonucleic acid and the absence of deoxyribonucleic acid have been assumed on the basis of results of colorimetric reactions distinguishing between pentose and deoxypentose. While at present there is little reason to suspect that plant viruses contain sugars other than ribose, it must be remembered that this has been rigorously demonstrated in very few cases.

The contents of individual purine and pyrimidine bases have been determined for the nucleic acids of several plant viruses and are summarized in Table V. As is shown in the table, and as was indicated by the earliest analyses of this sort by Markham and Smith (1951), the nucleic acids of different viruses contain demonstrably different proportions of the purine and pyrimidine bases and hence of the nucleotides containing these bases. In contrast, 15 strains of TMV were found to have nucleic acids so similar in composition that they could not be distinguished from one another (Knight, 1952; Black and Knight, 1953). These were the strains whose symptoms are illustrated in Fig. 2. Similar results were obtained on 2 strains of TMV by Cooper and Loring (1954). Actually, the earlier analyses of Markham and Smith (1950) indicate a great similarity among strains of TMV, except that their analyses on the type strain appear now to have been in error, and CV4 was included as a strain of TMV, whereas there is now some doubt that it belongs in the TMV group, at least in the sense that the above 15 strains do. Since the results of the analyses on different strains agree as closely as separate analyses on the same strain, the results have been averaged for Table V. (Certain corrections have been applied, as indicated in the footnote to the table, to give, it is believed, as accurate an estimate as present data permit.)

The nucleic acids of the 3 strains of tomato bushy stunt virus, whose symptoms are illustrated in Fig. 4, were also analyzed for their content of the purine and pyrimidine bases usually found in ribonucleic acids. These strains of BSV, like those of TMV, showed no significant differences in nucleic acid composition. Hence, an average figure for the three strains is given in Table V.

Markham (1953) has analyzed the nucleic acids of 7 strains of potato virus X and reports them to be very similar in composition, although the actual analytical data are not presented.

*b. Summary of Compositions of Strain Nucleic Acids.* All plant viruses analyzed so far appear to contain ribonucleic acid. Each different plant virus

TABLE V  
NUCLEOTIDE PROPORTIONS OF SOME PLANT VIRUS NUCLEIC ACIDS

| Virus <sup>a</sup> | Molar proportions of the nucleotides<br>in an arbitrary total of 4 |                  |                   |                  | References                            |
|--------------------|--|------------------|-------------------|------------------|---------------------------------------|
|                    | Adenylic<br>acid   | Guanylic<br>acid | Cytidylic<br>acid | Uridylic<br>acid |                                       |
| TMV and 15 strains | 1.13   | 1.00             | 0.77              | 1.11             | Knight (1954) <sup>b</sup>            |
| TMV                | 1.12   | 0.96             | 0.80              | 1.12             | Cooper and Loring (1954)              |
| CV3 and CV4        | 0.97   | 1.02             | 0.78              | 1.25             | Knight (1954) <sup>b</sup>            |
| BSV and 2 strains  | 1.02   | 1.12             | 0.82              | 1.03             | de Fremery and Knight (1955)          |
| TYMV               | 0.91   | 0.69             | 1.53              | 0.89             | Markham and Smith (1951)              |
| SBMV               | 0.97   | 1.03             | 0.96              | 1.05             | Dorner and Knight (1953) <sup>b</sup> |
| PXV                | 1.29   | 0.87             | 0.95              | 0.89             | Dorner and Knight (1953) <sup>b</sup> |

<sup>a</sup> The following abbreviations are used: TMV, tobacco mosaic virus; CV3 and CV4, cucumber viruses 3 and 4; BSV, tomato bushy stunt virus; TYMV, turnip yellow mosaic virus, SBMV, southern bean mosaic virus; PXV, potato virus X.

<sup>b</sup> The compiled figures used by Knight (1954) and by Dorner and Knight (1953) were corrected for an estimated 5 % hydrolysis of the pyrimidine nucleotides and the equivalent spurious increase of the adenine values, owing to an overlap of cytidine and adenine in the chromatographic step. Such corrections were made at the time of analysis for BSV and TYMV.

has been found to possess a nucleic acid containing characteristic proportions of purine and pyrimidine nucleotides. Thus, the nucleic acids of different plant viruses have demonstrably different compositions. In contrast, variant

strains of TMV, BSV, and probably of PXV have been found in each case to have nucleic acids of apparently identical composition. Hence, it has been suggested that identity of compositions of the nucleic acids of two viruses can be taken as evidence for strain relationship.

## 2. Structural Features of Strain Nucleic Acids

If, as present data suggest, strains of plant viruses do contain nucleic acids of the same composition, these nucleic acids could conceivably be different in function if the nucleotides were arranged in different sequences. A difficulty lies in the current lack of a systematic procedure for determining the sequence of nucleotides in polynucleotide chains. However, a method for probing possible structural differences is provided by the use of the enzyme, ribonuclease. Pancreatic ribonuclease has been shown to be a highly specific phosphodiesterase, which will hydrolyze secondary phosphate esters of pyrimidine riboside 3'-phosphates, but not of purine riboside phosphates. Two nucleic acids of the same composition, but having different sequences of purine and pyrimidine nucleotides, should therefore yield different products when digested with pancreatic ribonuclease.

a. *Strains of TMV.* As a beginning, the composition was determined of the residual (trichloroacetic acid-precipitable) material after ribonuclease digestion of the nucleic acids of each of 5 strains of TMV (Reddi and Knight, 1956). However, within the limits of the method of analysis used, the acid-insoluble ribonuclease-resistant residues of all 5 strains were found to have the same composition.

Turning to the acid-soluble fractions of the ribonuclease digests of strain nucleic acids, quantitative estimations were made of the mononucleotides present (Reddi, 1957). Only the pyrimidine nucleotides, cytidylic and uridylic acids were found. This was to be expected from the known specificity of pancreatic ribonuclease. As shown in Table VI, the amounts of cytidylic and

TABLE VI

AMOUNTS OF CYTIDYLIC AND URIDYLIC ACIDS RELEASED FROM NUCLEIC ACIDS OF SOME STRAINS OF TMV BY 12-HOUR DIGESTION WITH PANCREATIC RIBONUCLEASE AT 23°C. AND AT PH 7.6<sup>a</sup>

| Strain | Micrograms cytidylic acid/mg. nucleic acid | Micrograms uridylic acid /mg. nucleic acid |
|--------|--|--|
| TMV    | 80   | 128  |
| HR     | 74   | 121  |
| YA     | 79   | 124  |
| M      | 107  | 178  |

<sup>a</sup> Adapted from Reddi, 1957.



uridylic acid found were large, accounting for from approximately 42 to 71 % of the total amount of these nucleotides in the nucleic acids. This means that in a high proportion of cases two or more pyrimidine nucleotides are linked together in the polynucleotide chains without intervening purine nucleotides. Conversely, one can conclude that purine nucleotides must also frequently occur in clusters in the polynucleotide chains. However, the most significant fact revealed by the data in Table VI is the significantly greater amounts of cytidylic and uridylic acids in digests of *M* nucleic acid than in digests of the nucleic acids of TMV, HR, and YA. This is taken to be a clear indication of a larger proportion of pyrimidine polynucleotide segments in *M* nucleic acid than in the nucleic acids of TMV, HR, and YA, and constitutes the first evidence for structural differences between the nucleic acids of strains of a virus. Subsequently (Reddi, 1958), it has been found that ribonuclease digests of the nucleic acids of strains TMV, M, and HR contain different quantities of certain di- and tri-nucleotides. This provides additional evidence for the occurrence of different sequences of nucleotides in the strain nucleic acids.

*b. Summary of Structural Features of Strain Nucleic Acids.* Nothing has been found so far to distinguish viral ribonucleic acids from others of this type. Hence, it can be assumed that strain nucleic acids possess a common sugar-phosphate backbone that achieves unique quality by variation of the sequence of attached purine and pyrimidine bases, i.e., by different nucleotide sequences. Evidence for actual differences in nucleotide sequences has been found with three strains of TMV. This type of investigation is just beginning and should yield much more information in the next few years.

#### IV. PRODUCTION OF VARIANTS BY CHEMICAL AND PHYSICAL TREATMENTS

The knowledge that treatment with certain chemicals or exposure to radiations could cause genetic changes in higher organisms suggests that similar effects might be obtained with viruses. This seemed particularly likely with plant viruses, since they are chemically and morphologically so simple that they have often been considered raw genetic material akin to chromosomes. Consequently, the chemical structure of TMV (on which most of this type of experimenting has been done) has been modified by more or less specific treatments and the effects on biological activity observed. Likewise, the virus has been irradiated under a variety of conditions and the effects noted. None of these treatments has given results which could be unequivocally construed as production of mutants, but some of the treatments are briefly summarized here as a guide to further investigation along these lines.

### A. Chemical Derivatives of TMV

The two major components of plant viruses, protein and ribonucleic acid, both contain reactive groups which can be substituted, altered, or otherwise modified by treatment with specific chemical reagents. It was of considerable interest, first, whether or not significant changes could be made without completely inactivating the virus, and second, whether or not the modifications would be inheritable.

TMV has been treated with such reagents as formaldehyde (Ross and Stanley, 1938; Fraenkel-Conrat, 1954; Cartwright *et al.*, 1956), iodine (Anson and Stanley, 1941; Fraenkel-Conrat, 1955), ketene, phenyl isocyanate, carbobenzoxy chloride, *p*-chlorobenzoyl chloride, benzenesulfonyl chloride (Miller and Stanley, 1941, 1942), fluorodinitrobenzene (Knight, 1951), *N*-carboxyleucine anhydride (Fraenkel-Conrat, 1953), and benzyl and *n*-butyl mustards (Wood *et al.*, 1948).

It was possible to get modified but infective virus in all of these cases, but the disease caused by the chemical derivatives was indistinguishable from that of untreated virus and the virus produced was also the same as the untreated; that is, none of the changes was inheritable.

Another type of chemical modification is that caused by the enzymatic removal of the *C*-terminal threonine residues from the peptide chains of TMV and of 12 of its strains (Harris and Knight, 1955; Knight, 1955a). In the case of TMV, which was most extensively studied, this kind of reaction caused demonstrable changes in electrophoretic mobility and serological reactivity, but resulted neither in measurable change in infectivity nor in symptoms caused in several hosts. Moreover, the progeny from "dethreoninated" virus were found to have the *C*-terminal characteristics of untreated virus. In the 12 strains of TMV tested, no alteration of the symptoms in Turkish tobacco was caused by removal of the *C*-terminal amino acid residues.

With increasing evidence for association of genetic function with nucleic acid (see Chapter VI, Vol. I) it can be reasoned that the above chemical and enzymatic reactions failed to produce mutants because they involved primarily the protein components. There is, of course, evidence that the formaldehyde and mustards reacted with the nucleic acid of TMV as well as with the protein, but they may not have caused the type of change necessary for a mutation. Hence, considerable interest is focused on attempts to cause more deep-seated changes in the nucleic acid. In this connection, it has been possible to introduce substituted purines and pyrimidines *in vivo* into TMV and TYMV nucleic acids (Matthews and Smith, 1955; Gordon and Stachelin, 1958), but there is no indication that mutants were obtained thereby, unless the lesser infectivities of the modified viruses are interpreted as evidence for

lethal mutants. Likewise, the incorporation of 5-bromouracil into the deoxyribonucleic acid of T2 bacteriophage has proved to be a nonheritable change (Litman and Pardee, 1956). However, it was observed that unusually large numbers of plaque-type mutants resulted from infecting cells with T2 in the presence of sulfanilamide and 5-bromouracil; this was attributed to a disturbance of nucleic acid metabolism.

### *B. Relation of Radiations to Production of Variants*

Investigation of the effects of radiation on plant viruses have largely been concerned with inactivating doses of radiation (Pollard, 1954; Kleczkowski, (1957), but some irradiation experiments have been directed towards the production of mutants. Gowen (1941) reported that exposure of preparations of ordinary TMV and of the aucuba mosaic strain to low-energy X-rays increased the frequency of mutants observed in subsequent tests in plants. However, Gowen's data show that the X-ray treatment caused substantial inactivation of the viruses and that the two strains were not equally resistant to the radiation. Hence, the occurrence of a selective phenomenon rather than mutagenesis is a possibility which does not seem excluded, particularly since the data presented also show that unirradiated samples of each strain contained some of the other strain. Moreover, the lesion counts showing changes in proportions of the strains with increased exposure to radiation were not made on the same host, but on two different hosts of unequal sensitivity, thus compounding the normal difficulties of quantitative plant virus assays. Other workers (Kausche and Stubbe, 1940; Pfankuch *et al.*, 1940) have concluded that mutants were not produced by treatment of purified TMV with X-rays or gamma rays, although it was reported that irradiation of the infected plants caused an increase in mutation. Likewise, attempts to produce mutants by irradiation of bacteriophages (where quantitative detection methods are much better than with viruses of higher plants) have thus far been fruitless, although here again, irradiation of the host may be mutagenic (Laterjet, 1949).

Thus, at present, it must be concluded that mutations are not produced in plant viruses by treatment *in vitro* with chemicals or by irradiation. It is clear, however, that investigation along these lines has not been extensive and that the discovery of infectious preparations of virus nucleic acids may provide new material with which to make a fresh approach.<sup>1</sup>

## V. SUMMARY AND PERSPECTIVES

It is abundantly evident that plant viruses mutate to yield a variety of variant strains. These strains are characterized by diverse biological properties, usually best manifested by the types of disease symptoms they cause in

<sup>1</sup> Recently, Gierer and Mundry (1958) have reported production of mutants by treating TMV or its RNA with nitrous acid.

appropriate hosts. It is assumed that the different biological properties of strains are a reflection of different chemical structures and, conversely, that their similar biological properties have a common chemical basis. Common chemical characteristics have actually been found for plant virus strains. For example, strains of TMV have been found to have particles of the same size and shape; they have been found to possess the same proportions of protein and nucleic acid, the same kinds and proportions of nucleotides, the same numbers of peptide chains (viral subunits), and the same kinds of endings of the peptide chains. On the other hand, distinct chemical differences have also been found among the strains. In some cases, the strains differ in proportions of amino acids present and, in some cases, even in kinds of amino acids. This in turn leads to some different sequences of amino acid residues in the peptide chains. In some instances the nucleic acids of strains of TMV have been found to have differences in nucleotide sequence. Thus, there are pronounced chemical similarities in plant virus strains and there are distinctive chemical differences.

It seems almost certain that these similarities and differences in chemical structure are related to the similarities and differences in biological properties exhibited by the strains. In other words, it can be suggested that a chemical basis for the hereditary properties of these viruses is being established. A vital question in this regard is whether or not the whole nucleoprotein is involved in the genetic function. At present, evidence seems to favor a genetic role for the nucleic acid alone. If this is true, then the protein component of each virus is an even more direct expression of the genetic function of the virus than the biological properties as expressed, for example, in symptomatology. On this basis, it would be postulated that the various strains of a virus possess unique nucleotide sequences in their RNA components. These specific nucleotide sequences in turn furnish the patterns resulting in the synthesis of characteristic protein components. Evidence for the existence of such a situation is now being sought experimentally by attempting to determine the precise nucleotide sequences in the nucleic acids and the amino acid sequences of the protein components of several strains. Another obvious approach is the attempt to alter the nucleotide sequences in infectious nucleic acid preparations and to note the results. In any case, it seems that virus strains provide unique material with which to explore the chemical basis of heredity, for they represent at present the only isolable, homogeneous genetic substances available.

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## Chapter IV

### Biological Cycles of Plant Viruses in Insect Vectors

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#### I. INTRODUCTION

In 1931, Huff proposed that the transmission of parasites by arthropods be differentiated into four categories, three biological and one mechanical. The first biological type he termed *cyclo-propagative* and characterized it as including organisms that undergo both cyclical change, that is, morphological transformation to a different form, and multiplication in the arthropod vector (carrier). The second type, *cyclo-developmental*, encompassed parasites which change to a different form in the arthropod disseminator but do not multiply in it. In his third type, *propagative*, he included organisms which undergo no change in form in the arthropod vector but which multiply in it. Transmission of parasites which undergo neither change in form nor multiplication in the arthropod carrier he classed as *mechanical*.

Probably most, if not all plant virologists, would consider those plant viruses which multiply in their insect vectors as the only ones having a true biological cycle in their vectors. It is with these that we shall be principally concerned. From what is known about virus multiplication in general, it is to be expected that multiplication in the arthropod vector would involve a change in the form of the virus during the process of multiplication itself. We know something about the form of the end products of this multiplication in both plant and insect in only one case, that of wound tumor virus (Brakke *et al.*, 1954; Black and Brakke, 1954). Here the evidence indicates that both the virus and viral soluble antigen are the same in both hosts. We, therefore, have reason for supposing that the process of multiplication in the insect is essentially the same as that in the plant. On the basis of present knowledge, the plant viruses with biological cycles would therefore belong in Huff's

*propagative* category. This term will therefore be used to designate both these viruses and their kind of transmission.

It is impossible to deal adequately with the propagative plant viruses without also considering evidence concerning another type of relationship in which the plant virus is taken in through the mouth parts, accumulated internally, apparently without multiplication, passed through the insect tissues, and introduced into plants again via the mouth parts of the carrier. The most thoroughly studied virus of this kind is that causing sugar beet curly top. In this paper such viruses and the relationship they exhibit will be called *circulative* because of their remarkable ability to complete this circle of penetration through the membranes of the body of the vector without multiplication. It is realized that the propagative viruses have both circulative and propagative properties and that the terms are not mutually exclusive. However, they are not cumbersome and they do emphasize the distinctive feature of each class. Other names for circulative transmission have been proposed earlier. The circulative viruses belong in Huff's *mechanical* category. However, the term "mechanical" seems inadequate both descriptively and as a means of distinguishing such transmission from other very different mechanical types. Freitag (1936, p. 340) described the vectors of curly top as "internal mechanical carriers." The term "vector-latent" of Day and Rzykiewicz (1954, p. 270) apparently would include both circulative and propagative viruses.

In order to give the reader some orientation, other kinds of relationships between plant viruses and their vectors will be mentioned here but will not be discussed. The most important of the other kinds of relationship is that shown by the nonpersistent, aphid-borne viruses, which constitute the largest group of plant viruses. Recently, Bradley and Ganong (1955, 1957) demonstrated that the transmitted particles of a number of these viruses are carried upon the tip of the stylets—more exactly, the distal  $15\ \mu$ . The nonpersistent, aphid-borne viruses are obviously noncirculative, but there are some aphid-borne viruses whose position between the two extremes of propagative and nonpersistent is uncertain and whose manner of transmission is not clearly understood. Beetles, with biting mouth parts, transmit a number of viruses that cannot be transmitted by the sucking mouth parts of aphids. There is evidence that regurgitation of virus-containing juice may be important in virus transmission by such beetles. It has been generally considered that transmission by beetles is purely mechanical, but the demonstration that virus may occur in the blood, that transmission may occur 20 days after the end of acquisition of virus and even after over-wintering of the beetles, indicates that, in some cases at least, it is possibly circulative (Freitag, 1956). Simple mechanical transmission is exemplified by grasshopper dispersal of potato spindle tuber or tobacco mosaic virus (Goss, 1931; Walters,

1952) in which the virus is probably merely mechanically transferred by the biting mouth parts. Recently an unusual kind of mechanical transmission was indicated by the evidence that the ovipositors of certain leaf miner flies may transfer tobacco mosaic and sowbane mosaic viruses (Costa *et al.*, 1958).

There are a number of soil-borne plant viruses whose means of survival in the soil and transmission from plant to plant has been a tantalizing mystery for many years. For this reason the recent discovery by Hewitt *et al.* (1958) that the ectoparasitic nematode, *Xiphinema index*, transmits the soil-borne grape fanleaf virus is of great interest. The potential significance of this finding is difficult to assess at this time but it may well herald future discoveries of many nematode vectors with new intriguing relationships to the viruses they carry. It has definitely demonstrated how some soil-borne viruses are spread.

Before entering upon the discussion of the main theme of this paper, a few additional introductory remarks are in order regarding two broad generalizations on arthropod transmission of plant viruses. One is that the vectors of a single plant virus are almost always restricted to one of the major taxa, such as that comprising the aphids, the leafhoppers, the white flies, the beetles, the thrips, the mealy bugs, or the mites. Most records to the contrary in the literature are not well authenticated and others could well be reinvestigated in the light of the bulk of evidence supporting the rule. The second generalization is that a virus is almost always transmitted by only one of the principal types of transmission, that is, by propagative transmission, by circulative transmission, or by one of the mechanical processes, simple or not so simple. However, there are indications that some of the aphid-borne and some of the beetle-borne viruses are transmitted in more than one way. Even if this should prove to be true, this second rule has wide validity.

## II. THE NATURE OF THE INSECTS AND VIRUSES INVOLVED IN BIOLOGICAL CYCLES

The problem of ascertaining whether or not any plant viruses have a biological or reproductive cycle in their insect vectors was worked out with those plant viruses that are transmitted by leafhoppers and the reader needs to be aware of certain characteristics of both the leafhoppers and the viruses they carry in order to understand the necessity for certain experimental procedures used in investigations. Leafhoppers are small insects, weighing about 1 or 2 mg., which live by sucking nutriment, usually from the vascular structures of plants. They insert their tubelike mouth parts and commonly withdraw plant juices from the phloem, but some of them feed on the xylem. Although some may be maintained for a few days on artificial nutrient solutions, no one has discovered how to rear them on such solutions. Thus



although the adults may fly great distances, they must be treated like obligate parasites, a fact which severely restricts the experimental procedures that can be used. In order to keep the insects alive during a test for virus increase they must be maintained upon suitable host plants. At the same time it is necessary to ensure that they are not obtaining increments of virus from these plants. This can be accomplished by using plants insusceptible to the virus or by using susceptible plants under special conditions. Inoculated plants undergo an incubation period of at least 2 days in some cases to almost a month in others (Kunkel, 1948, p. 42). The leafhoppers cannot acquire virus from plants until shortly before the symptoms develop so that daily transfers or frequent transfers may prevent acquisition of virus from susceptible plants (Maramorosch, 1953b, p. 799). Another special condition for using susceptible plants is provided by the long incubation period of the virus in the vector. This is the period between the time of acquisition of virus and the time the insect is able to inoculate plants with it. In cases in which this is long, for example, two weeks, it may be possible to measure virus increase while the insects are maintained on very susceptible plants, because the subsequent failure of any of the plants to develop disease assures that no virus could have been secured from them (Black, 1941). The elimination of weeds from the cages is, of course, an elementary precaution.

The reproductive habits of leafhoppers also have a bearing upon the design of experiments. Sometime after mating, the female inserts her eggs into the leaf or stem of a plant by means of her ovipositer; about a week or more elapses before the eggs hatch and the small wingless nymphs emerge. At times, as the nymph grows, it sloughs off its entire hard chitinous skin or exoskeleton, including the lining of the mouth parts, and fore- and hind-gut. This process usually occurs five times before it reaches the adult or winged stage. Wing pads and the external male and female genitalia are apparent before the insects become adults. The fact that the eggs undergo development to the nymphal stage while inserted inside and protected by plant tissues means that, unless precautions are taken, young vector insects may emerge in the greenhouse from eggs inserted into plants by inoculating insects, may acquire virus, and may subsequently spread it to other plants. Such secondary, uncontrolled spread must be avoided. This can be accomplished by using only males in the tests or by separating the sexes in the nymphal stage. If the incubation period in the insect is more than a week long, it is simpler to use both males and females indiscriminately and, by fumigation once a week, to kill hatched nymphs before they are able to inoculate plants.

Besides being transmitted specifically in nature by one or a number of related leafhopper vectors, the propagative viruses also have their peculiar and rather exasperating traits. Most of these viruses are very unstable outside the living cell. With one or two exceptions none of them can be successfully

inoculated directly into plants. Extracts from plants, known to contain virus, cannot, in most cases, be successfully used to inoculate very susceptible plants. The few exceptions have not helped in the solution of the question about biological cycles, because in none of these cases nor in any others has a leafhopper virus in an extract of the vector been successfully inoculated into plants. Yet until very recently the only methods of detecting such a virus or measuring changes in its concentration depended upon obtaining infections in plants. No virus assay by serology, electron microscopy, or by disease effects in the vector was available for any of the viruses under investigation.

### III. HISTORY OF RESEARCH ON THE PROBLEM

Research on the problem of multiplication of plant viruses in their insect vectors has been carried out in various parts of the world on viruses that scientists were afraid to ship freely from one country to another because of their possible devastating effects on crops. This meant that men in one part of the world, or even in two different parts of a single country like the United States, worked on one virus that they considered appropriate, and men elsewhere chose another. The subject was healthily controversial; Bawden, in England, played a stimulating role as a critic of some of the work. However, neither he nor his colleagues in Western Europe were able to work on the problem, because no leafhopper transmission of virus was discovered in Western Europe until de Fluiter and his colleagues (1955) in Holland successfully obtained transmission of *Rubus* stunt virus with *Macropsis fuscula* in June, 1953.

The multiplication of certain plant viruses in their insect vectors, as well as in the plants they infect, is now generally accepted. In fact, it now seems that the probability of virus multiplication in certain vectors of plant viruses is sometimes too readily accepted and that ideas that were advanced during the controversy over the problem are sometimes forgotten. Some of them may, however, merely be in eclipse and will prove eventually to have a place in the total virus picture.

As early as 1915, Smith and Bonquet had found that the beet leafhopper, after acquiring curly top virus from plants, required an incubation period of 24 hours before the insects could infect healthy plants. They thought the causal agent underwent some development or change in the insect before it could be transmitted.

In 1926, Kunkel published a comprehensive account of his studies on aster yellows. Kunkel was impressed by the fact that, after aster leafhoppers had acquired the causal virus by feeding on plants with yellows, a period of at least 10 days intervened before the leafhoppers were able to infect healthy plants. However, once the insects became infective, they usually continued to be so during the remainder of their lives, in some cases for as long as 100

days. The virus did not pass through the insect egg. If the insect acquired virus during the nymphal stage only, the virus persisted in the insect, even when it underwent molts which must have removed virus from all external parts, including the mouth parts. By moving them to a fresh, healthy plant each day or by maintaining them on rye, which he showed was not susceptible to the virus, Kunkel made sure that the insects did not pick up fresh virus after molting. These facts clearly indicated that the virus was carried inside the insect, and Kunkel concluded "It seems probable that the incubation period of aster yellows in *Macrosteles fascifrons*<sup>1</sup> is due to a development and multiplication of the causative agent in some tissue of the leafhopper."

Kunkel looked for some difference between the viruliferous and non-viruliferous leafhoppers but found none. The average length of life of the virus-bearing and virus-free vectors was the same. He also sought for microscopic evidence of disease in the infective insects but was unable to find any.

Storey (1928), working with corn streak virus in Africa, found that the minimum incubation period in the vector *Cicadulina mbila* was 6 to 12 hours and that once insects were infective they retained infectivity, when transferred daily, throughout their lives, although occasional exceptions occurred. Infective leafhoppers captured as soon as a nymphal skin was shed were still infective. Storey (1928, p. 22) believed "the evidence clearly indicates a multiplication of the virus in the insect."

In 1933, Storey transmitted corn streak virus extracted from plants or from vectors by introducing it into nonviruliferous *Cicadulina mbila* by means of a needle or glass micropipette. Using this technique to test for virus he determined that the virus occurred in the blood before the end of the incubation period. He also detected it in the general contents of the thorax and abdomen, but in the contents of the rectum, only if the insect had recently fed on a diseased plant. It did not occur in the naturally voided feces.

In the same year, Fukushi (1933) reported the first authentic and well-demonstrated instance of plant virus transmission through the egg of the vector. According to Fukushi, the Japanese entomologists, Onuku and Murata, had discovered, about 1902, that leafhoppers (*Nephotettix apicalis* var. *cincticeps*) which hatched from eggs collected in Shiga prefecture produced stunt (dwarf) disease in rice plants, whereas leafhoppers of the same species from the vicinity of Tokyo did not. They subsequently found that non-infective leafhoppers from Tokyo became infective after feeding upon diseased plants. According to Fukushi, Murata stated in 1915 that infectivity was transmitted from parents to progeny for 3 or 4 generations. Fukushi stated that no experimental procedures or results were reported in detail to support

<sup>1</sup> Currently accepted binomials are used throughout and have been substituted for the originals in quotations.

these conclusions. According to Fukushi, the entomologists at that time were unaware of the virus nature of the disease and considered that the ability to produce dwarf disease in rice was a character specific to the leafhoppers native to Shiga prefecture. Fukushi, on the other hand, convincingly demonstrated that the disease-producing agent was transmitted to the progeny when the female was viruliferous and the male nonviruliferous, but not when the female was nonviruliferous and the male viruliferous—in other words, that the virus passed through the egg but not through the sperm. He also determined that nymphs hatching from eggs laid by noninfective females in diseased plants were nonviruliferous. From these experiments, Fukushi concluded that the virus entered the eggs in the ovary.

Later, Fukushi (1934) reported that, in spite of the fact that this virus was regularly transmitted through the egg and that inoculation feedings as short as 5 minutes were sometimes successful, extensive tests indicated that the virus was, sometimes at least, difficult for the vector to acquire. For example, in one set of acquisition feeding periods varying from 3 to 50 days, only 25 of 1300 leafhoppers were demonstrated to have acquired the virus. In such insects the incubation period varied from 10 days to 2 months. Nymphs which acquired the virus transovarially sometimes transmitted the virus immediately after emergence, but usually an incubation period of 1 to 14 days elapsed before newborn nymphs became infective.

In the same paper, Fukushi reported that virus inclusion bodies occurred in the chlorotic tissue of a number of grass species affected by rice stunt. However, although he examined thousands of sections, he was unable to find any such bodies or any other change of etiological significance in the salivary glands, alimentary canal, egg follicles, ovarian tubules, mycetome, or other organs of viruliferous vectors. If either Kunkel in 1926 or Fukushi in 1934 had been able to detect any deleterious effects of the virus upon the vector, it seems likely that others would have been much more ready to accept the idea of plant virus multiplication in the insect vector.

In 1935, Fukushi reported his first experiment on the passage of rice stunt virus through the egg of its leafhopper vector to several succeeding generations without access to virus in diseased plants. A single viruliferous female was mated to a nonviruliferous male and allowed to oviposit eggs in rice plants. At the moment of hatching, the young nymphs, which averaged 0.06 mg. in weight, were removed from the plant in which the egg had been inserted and transferred to a healthy young rice seedling. This process required patient observation but ensured that the young insect had not sucked up virus from the plant on which its viruliferous mother had fed and oviposited. Subsequently, each nymph was transferred daily to a healthy young rice seedling throughout its life of 2 months or so, a procedure which ensured that the nymph could not acquire fresh virus from plants. Fukushi had obtained



evidence earlier (1934) that the insects did not acquire virus in less than 3 days of feeding on diseased plants. Moreover, insects in general are unable to acquire virus from plants until a day or two before plants develop symptoms; Fukushima (1934, pp. 85, 86, 90) had determined that the incubation period in plants varied from 6 to 37 days. In his experiment, one of the young nymphs, weighing about 0.06 mg., was a female that infected 38 plants. It also oviposited eggs from which the nymphs, handled in the same manner, included 15 infective progeny that infected 201 plants. Fukushima reasoned that the amount of virus in the original 0.06 mg. nymph must have been "extremely small" and that the passage of the virus to this nymph and to the next generation of progeny, and the infection of so many plants, meant that the virus multiplied in the vector.

One year later, Freitag (1936) published his studies with the curly top virus in sugar beets and interpreted his data as evidence that the virus did not multiply in the beet leafhopper, *Circulifer tenellus*. In this case the insects never receive virus through the egg. After allowing the leafhoppers to acquire virus by feeding on diseased plants for various periods, he transferred individual insects to fresh healthy beet seedlings each day. This ensured that the only virus acquired from plants was that obtained in the initial acquisition feeding period. During successive equal intervals after the acquisition of virus, the percentage of beets infected by single insects tended to decrease progressively. In some cases infective ability was lost entirely. Moreover, the ability of the leafhoppers to infect was roughly proportional to the length of the acquisition feeding period. Although the ability of adult insects to acquire and to transmit virus decreased with age, this effect was less marked than the progressive decrease in transmission with time, which occurred when insects were not allowed renewed access to virus. In other words, the age effect could not entirely account for the decrease. Furthermore, it was demonstrated that a second feeding on a source of virus in late adult life increased the infectivity of insects that were transmitting poorly. Freitag reasoned that, if multiplication occurs in the vector, the insects should not only retain infective capacity during their entire adult life, but that those fed for only a short period on diseased beets should be able to cause as many infections as those fed for longer periods. He considered that the virus did not multiply in the insects.

Not long afterwards, Kunkel (1937) published his first paper on the effect of heat on the ability of *Macrostelus fasciatus* to transmit aster yellows virus. He showed that infective leafhoppers, after being kept at approximately 32°C. for varying numbers of days, were unable to transmit the virus when returned to temperatures of about 24°C. The period of their inability to transmit was roughly proportional to the length of time they were held at the high temperatures. If they were kept at the high temperatures for 12 days or longer they lost infective ability permanently, unless allowed another



acquisition feeding on diseased plants. Kunkel also demonstrated that, if insects were heat-treated while undergoing natural incubation periods, the effect of the high temperature was greater than if the insects were already infective. Kunkel believed that these results indicated that heat treatments of 12 days or more completely destroyed the virus in the insects, that heat treatments for shorter periods destroyed virus in proportion to the duration of the heat treatment, and that the time required for regaining infectivity represented an induced incubation period in which the virus must once more multiply to an infective concentration. The greater effect of the treatment on insects in which virus was still undergoing incubation, was attributed to a lower virus content in these insects when the heat treatments were started, than that in insects already infective.

Kunkel observed that the heat-treated insects frequently transmitted milder strains of the aster yellows virus and suggested that the heat treatment exerted some kind of selective action for mild strains of the virus. The observation indicated that the heat treatment was actually preferentially inactivating the more virulent strains of the virus and was in itself evidence for a direct effect on the aster yellows virus.

Before Kunkel's paper appeared, Bennett and Wallace (1938) had sent to press a report of their extensive investigation of curly top. Among other things, they found that, although the curly top virus could be acquired in as short a time as one minute of feeding, individual leafhoppers did not acquire their maximum ability to transmit unless the acquisition feeding lasted for two days. Acquisition feedings for longer than two days increased the virus concentration in the leafhoppers but did not increase ability to transmit. They were able to ascertain the latter point by preparing extracts of the leafhoppers, by allowing other nonviruliferous leafhoppers to feed on the extracts through a membrane, and by then testing the infective ability of these insects on beet seedlings. The essentials of this artificial feeding technique had been described by Carter (1927) and by Severin and Swezy (1928). Although infections can be obtained by multiple pin pricks through virus extracts into the crowns of beet plants, the successful inoculations are so rare and irregular that the method has never been used to assay curly top virus.

Subsequent to acquisition of virus and passage of the incubation period, Bennett and Wallace found that the infectivity of the leafhoppers and their virus content gradually decreased over periods of 8 to 10 weeks. However, reduction of infectivity was slight if the original virus content was high. Ability to transmit at a higher level of efficiency could be restored by renewed access to a diseased plant. Bennett and Wallace also determined the minimum incubation period in the leafhopper to be four hours. By the feeding technique described above they ascertained that the virus occurred in the blood, alimentary tract, salivary glands, and the feces. The blood was the richest

source and the salivary glands a relatively poor source. They considered that the salivary glands might be unfavorable to the virus.

It is apparent that their findings confirmed and extended those of Freitag. The authors considered it probable that there was no multiplication of curly top virus in its leafhopper vector and that, if multiplication occurred, it was insufficient to maintain the virus content of the vector. As a result of their work, Bennett and Wallace suggested that if the same conditions held generally for other insect vectors that retain viruses for long periods "much of the evidence now accepted as supporting the idea that certain plant viruses multiply in their insect vectors must be re-evaluated."

In support of his earlier work, Kunkel (1938) reported that newly hatched nymphs of the aster leafhopper can grow to the adult stage, produce fertile eggs, and otherwise live what appear to be normal lives when held continuously at 95°F., a temperature 5° higher than that used to cause temporary or permanent loss of infectivity. He demonstrated again that insects that have been rendered permanently noninfective by heat treatment may reacquire and transmit the virus by feeding on diseased plants. Because of these facts, and the fact that heat treatment of plants indicated inactivation of the virus in plants, Kunkel concluded that the effect of heat treatments on insect infectivity must be attributed to action on the virus. He considered that multiplication in the insect was the basis for the specific relationship existing between the aster leafhopper and the aster yellows virus.

In 1939, the first edition of Bawden's widely read and influential book, "Plant Viruses and Virus Diseases," was published. He accepted and strongly supported the conclusions of the workers on curly top virus and, although he considered that multiplication of plant viruses in vectors could not definitely be excluded, he advanced many arguments against the evidence that such multiplication occurred. It would be tedious to recapitulate here all the arguments that were advanced against multiplication of plant viruses in their insect vectors. Instead, only those ideas that still have pertinence in the light of our present knowledge will be dealt with. Bawden's opposition to the evidence for multiplication was continued through the 1943 and 1950 editions of his book, except for an addendum in the latter. Some of his criticisms were valid and are still valid today. For example, Bawden's important arguments regarding the possible number of virus particles which a single leafhopper might contain were actually extremely conservative, as will be seen later. On the other hand, some of the arguments advanced against multiplication were erroneous and some seem very involved and improbable, though tenable until proof to the contrary was forthcoming.

In 1939, Fukushi briefly reported further studies on the transmission of the rice stunt virus through the egg and, in 1940, he published complete details of

these experiments. In one case he obtained transovarial passage of virus to the sixth generation of progeny. The virus was retained for at least 374 days and infections were obtained in about 1200 rice plants by 82 infective leafhoppers derived from a single viruliferous female. One egg from this female gave rise in subsequent generations to leafhoppers that infected more than 1000 plants. No consistent or progressive reduction in the infective ability was detectable throughout the generations, and Fukushi concluded that, since the original amount of virus in a leafhopper, and especially in an egg, must have been extremely small, it seemed necessary to assume the multiplication of the virus in the leafhopper to account for the results. Usually, there was an incubation period of 7 to 38 days before newly hatched nymphs began to infect plants, and, after the insects became infective, they remained so for as long as 88 days. Fukushi considered it likely that the incubation period after hatching represented the time required for the virus to multiply in the insect. The frequency of transmission varied from that in a few viruliferous females, which infected no plants during their entire life but produced infective progeny, to that in others, which infected plants consistently on consecutive days. The infective capacity of some leafhoppers was reduced or lost, especially in old age.

In leafhoppers which did not transmit during their life, but which produced infective progeny, Fukushi suggested that the virus might have been inhibited in its multiplication and localized by chance in the ovarian tubules. He noticed that when nonviruliferous females were mated with viruliferous males, the virus-free progeny had a greater ability to acquire and transmit than did those derived from nonviruliferous parents. He also noted that an average of 85 % of the offspring of infective parents proved to be infective while, on the average, only 60 % of those derived from infective females and nonviruliferous males became infective. He postulated a dominant genetic factor for susceptibility in the leafhopper to explain these differences. Such a genetic factor was also suggested to explain the striking difference between the percentage (12 %) of infective progeny from nonviruliferous parents, that (68 %) from crosses between infective females and nonviruliferous males, and that (92 %) from infective males and infective females when such progeny were reared on dwarf diseased rice plants. Fukushi considered that the long incubation period in the leafhopper, varying from 10 to 73 days, was too long to represent the time necessary for virus merely to migrate through body fluids and tissues.

In 1941, Kunkel reported in detail his experiments on the cure of *Vinca rosea* and *Nicotiana rustica* plants infected with aster yellows. The treatments were carried out at higher temperatures, usually 38 to 42°C., and for longer periods of time, usually 2 or 3 weeks, than were necessary for the inactivation of the virus in leafhoppers. These experiments demonstrated that the virus

*in vivo* was very readily inactivated by high temperatures in plants as well as in insects, and supported Kunkel's interpretation of his work.

Black found that, by using Storey's (1933) technique of insect injection, he could transfer aster yellows virus from viruliferous to nonviruliferous leafhoppers. In 1941, he titrated the virus in samples of leafhoppers from a colony that had been allowed to feed on diseased plants for one day. After the acquisition period of one day, the colony, minus the samples that were used for titrations at different times, was subsequently maintained on healthy aster plants and transferred at frequent intervals. Counting the day in which the insect fed on diseased plants as 1, two samples of 50 insects each, were withdrawn at random from the colony on the 2nd, 4th, 8th, 12th, and 16th days and tested for virus at different dilutions. During this time the source insects were maintained on susceptible aster plants, none of which became diseased. It was impossible, therefore, for the insects to have introduced virus into these plants and to have withdrawn virus from them after it had multiplied in the plant host. From the 18th to 31st day the remainder of the colony infected the two fresh aster plants to which it was transferred each day. It was found that on the 2nd day no virus could be detected in insect juices diluted to 1 : 10 or 1 : 100, but on the 12th day, virus could be detected at 1 : 100 and 1 : 1000; Black interpreted his results as showing at least a 100-fold increase of virus in the vectors during the incubation period. Similar results were obtained by Maramorosch (1953a, 1956). Whitcomb and Black (to be published) have followed the increase of viral soluble antigen in leafhoppers of *Agallia constricta* after injection of wound-tumor virus. The leafhoppers were maintained at 27.5°C on alfalfa which is immune to the virus. By means of the precipitin ring test they demonstrated that no antigen could be detected at a dilution of 1/10 through the 4th day following the injection. Between the 5th and 8th day the titer of viral soluble antigen rose from 1/10 to 1/120 and after the 12th day it was maintained at a titer of 1/160 for at least 50 days. This curve resembles that for the increase of  $\sigma$  virus in *Drosophila melanogaster* (L'Heritier, 1958, p. 205).

In 1948, Kunkel pointed out that in three different viruses affecting corn there was a correlation between the length of the incubation period in the specific vectors and in the corn plant. He drew attention to the fact that this approximate correlation held for six other virus diseases. In regard to the viruses affecting corn, Kunkel pointed out that the three different vectors were about the same size and considered that there was no obvious reason why one of the viruses should require only one-half day to pass from gut to saliva, while another virus should require 14 days or 28 times as long to make a similar trip in another leafhopper. If physical passage of the virus from one location to the other alone were involved, he found it difficult to understand why the incubation periods in the corn plant should be approximately the



same as in the leafhopper, or in the same order as those in the leafhoppers. If, on the other hand, the incubation periods in the vectors represented, not only such passage from gut to saliva, but also periods of virus multiplication, then a rapidly multiplying virus could be expected to have a short incubation period in both vector and plant, and a slowly multiplying virus would be expected to have a long incubation period in both.

In each of the years 1939 to 1942 the writer used the insect injection technique in large-scale attempts to transmit aster yellows virus from insect to insect in series. All of these attempts were complete failures; the mortality of the insects in the second passage was very high and none of the few survivors was infective. At about this time a virus, clover club leaf virus, was found in *Agalliopsis novella* (Black, 1944), and it happened to be transmitted through the egg to a high percentage of the progeny (Black, 1948). It was chosen as a means for obtaining proof that a plant virus could increase in its insect vector (Black, 1950). A single viruliferous female weighing 1.7 mg. was mated to a nonviruliferous male and was allowed to oviposit its eggs in immune alfalfa plants. A portion of the progeny was tested on susceptible crimson clover to determine the proportion that was infective, and the remainder of the progeny were reared throughout their lives on alfalfa. From the proportion of insects that infected crimson clover the number of infective insects among all the progeny of the female was estimated; the reciprocal of this number constituted a conservative measure of the average dilution of the starting virus in each of the progeny.

Females among the progeny kept on alfalfa all their lives were mated with nonviruliferous males and the experiment continued in such a way that selection of the main line of descent was independent of virus concentration. After continuing the experiment for more than 5 years through 21 generations, it was calculated that the dilution of virus in the original female was equivalent to at least  $1/10^{26}$ , whereas the maximum number of virus particles that she could have contained was estimated to be  $10^{12}$ . The virus was also passed by graftage from one *Vinca rosea* plant to another through 30 passages in series. The experiment provided proof that this virus multiplied in both insect vector and plant host.

While the third edition of Bawden's book was in press he was shown these data when visiting the United States. As a result, he included an addendum in the 1950 edition agreeing with the evidence for the ability of this virus to maintain itself indefinitely in its insect vector.

Maramorosch (1952a), using the insect injection technique, succeeded in the serial transmission of aster yellows virus from leafhopper to leafhopper under conditions which assured no replenishment of virus from plants. The insects were maintained on immune plants or left on susceptible plants for only very short periods. At each transfer he inoculated the insects with a



known volume of inoculum at a high dilution, usually  $10^{-3}$ . He calculated that the dilution of the starting quantity of virus at each transmission was  $10^{-4}$  because the inoculum injected into each insect was approximately 1/10 its weight. After 10 passages in series, the dilution had reached  $10^{-40}$ , whereas ordinarily the virus from insects is infectious at dilutions of  $10^{-3}$ , but not  $10^{-4}$ . In fact, Maramorosch's starting dilution in this series, 1/7000, was the greatest dilution of aster yellows virus successfully inoculated up to that time. In retrospect, it seems probable that the high dilution of his inocula, as well as the optimum temperature at which the experiment was conducted, were important in the success of his experiment. This experiment proved that aster yellows virus multiplies in its vector. Maramorosch later (1955) demonstrated that this virus could be passed serially from vector to vector when the insects were maintained only on insusceptible plants.

Maramorosch told the writer of his success at an early stage in the conduct of his first experiment on serial passage of the virus through the vector and an attempt of the same sort was started with wound tumor virus and its vector *Agallia constricta*. The insects used as a continuing source of virus were always kept on immune plants. At each passage a sample was tested on susceptible plants and then discarded. The results (Black and Brakke, 1952) confirmed completely Maramorosch's conclusions that a plant virus could be transmitted in series from vector to vector without loss of titer under conditions which assured no replenishment of virus from plants. Similarly, the virus of corn stunt was transmitted at dilutions of  $10^{-2}$  from vector to vector through 3 serial passages (Maramorosch, 1952b).

In a critical review of the subject, Black (1953b) considered in detail the controls that were employed in various experiments up to that time and the interpretations and conclusions that had been made. Relevant objections that had been raised were considered in detail. The results of Fukushi's experiment were re-evaluated in the manner employed for the treatment of the data on clover club leaf; it was thereby demonstrated that the dilution of virus in the female with which Fukushi started his experiment was at least 1/563,000 and probably at least  $1/10^8$  in the sixth generation of progeny.

At that time, Black (1953b) estimated that the number of virus particles probably carried in a leafhopper ranged from a minimum of  $10^6$  to a maximum of  $10^{12}$ . It is now possible to give an estimate of the average number of particles of one virus, namely, wound tumor, that occurs in its vector. The titer of this virus in a group of infective leafhoppers was measured serologically by titrating the virus zone from rate density gradient centrifugation tubes. The end-point titer was about 1/320, or  $10^{-2.5}$  (Black and Brakke, to be published). The best estimates of the diameter of the virus give a value of 75  $\mu$ . This is based on measurements of electron micrographs of the purest preparations of the virus obtained (Brakke *et al.*, 1954). The volume of the

wound tumor virus particle is approximately  $10^{-12.7}$  cubic millimeters. Merrill (1936) assumed a specific gravity of 1.0 for all antigens considered in deriving his generalization relating number of virus particles per cubic centimeter at serological thresholds and the mass of each particle. On the same assumption, the mass of each wound tumor particle would be  $10^{-12.7}$  mg., and it can be estimated from Merrill's experimental curve (1936, Fig. 1) that, at the end-point titer, a solution of wound tumor virus would contain  $10^{10}$  virus particles per milliliter. However, Merrill was dealing with end points determined by mixing antigen and antiserum solutions, whereas Black and Brakke were using the ring test. This gives approximately tenfold higher titers. Therefore, the end-point solution containing  $10^{-2.5}$  gm. leafhoppers per milliliter is estimated to contain  $10^9$  wound tumor virus particles. Adult leafhoppers weigh about 2 mg., so that the average leafhopper would contain  $\frac{2 \times 10^9}{10^{-2.5} \times 10^3} = 10^{8.8}$ , or about  $10^9$  virus particles. This figure is halfway between the maximum and minimum probable numbers estimated by Black (1950, 1953b).

It may well be, of course, that the average number of particles of rice stunt virus in its vector is much less. Wolcyrz and Black (to be published) were unable to detect potato yellow-dwarf virus in extracts of its vector, *Aceratagallia sanguinolenta*, at dilutions of 1/16. When this virus is photographed under the electron microscope it rarely assumes a roughly spherical shape. When it does, its diameter is about  $130 \text{ m}\mu$  (Brakke and Vatter in Black (1955), Fig. 4). By calculations similar to those above, it may be estimated that the number of particles of potato yellow dwarf virus per leafhopper is less than  $10^8$ .

Black (1953b) concluded that the multiplication of rice stunt virus, clover club leaf virus, aster yellows virus, and wound tumor virus in their vectors had been demonstrated. The evidence for the propagative relationship between some plant viruses and their leafhopper vectors has continued to accumulate.

Kunkel (1954) studied groups of about 50 *Macrostes fascifrons* fed for 2 hours, 1 day, 7 days, or 14 days on asters with yellows, and subsequently tested individually on a fresh aster plant each day as long as they lived. Kunkel considered that the insects that did not live for 26 days or more after the start of virus acquisition had not been adequately tested. Among the others, 6/47, 47/68, 42/50, 53/53, or 13 %, 69 %, 84 %, and 100 %, transmitted in the 1/12-, 1-, 7-, and 14-day groups. If one considers only the plants that were used in the tests after each individual began to transmit, the percentages of plants infected by infective insects in the four groups were 71, 72, 75, and 78. This is a remarkably uniform result, consistent with the multiplication of this virus in its vector. Kunkel also found that a

considerable number of the insects remained infective as long as they lived.

Additional instances of transovarial passage have been recorded; in the case of rice stripe virus transmitted by *Delphacodes striatella*, Yamada and Yamamoto (1955, 1956) have shown that the virus may pass transovarially to 23 generations of progeny under conditions which indicate multiplication of the virus in the vector. The insects varied genetically in their ability to transmit; the greatest, least, and intermediate proportion of infective progeny occurred among descendants of the active (susceptible) insects, inactive insects, and crosses between the two, respectively. Nymphs from females which had just acquired virus before ovipositing required an incubation period before transmitting; those from infective females did not. As in the case of rice stunt virus and clover club leaf virus, the virus did not pass with the sperm and almost all progeny of infective mothers received virus.

Transovarial passage of certain other viruses has been measured as 42 % for rugose leaf curl virus (Grylls, 1954), 2-11 % for wound tumor virus, and 1 % for New Jersey potato yellow dwarf virus (Black, 1953a).

Although the Californian aster yellows and the typical aster yellows of eastern United States had been distinguished by their host ranges since 1932, it remained for Kunkel, in 1955, to distinguish the two diseases on the basis of their symptoms. This enabled him to demonstrate for the first time that the presence of one plant virus in a leafhopper may interfere with the transmission of another. Colonies of *Macrostes fascifrons*, allowed to acquire one of these viruses during a feeding period of two weeks, were unable to transmit the other virus when given ample opportunity to do so. The same cross-protection was later demonstrated with individual leafhoppers (Kunkel, 1957). This result is in marked contrast to the earlier findings of Giddings (1950) using strains 2 and 3 of curly top virus; Giddings found there was no cross-protection.

Incidentally, this reaction in the leafhopper provides a means, not previously available, for testing relationships between viruses and, in some cases, it might provide the only means available at present. For example, *Colladonus geminatus* is a vector of California aster yellows (Severin, 1934) and several strains of cherry buckskin virus (Jensen, 1956) and it may therefore provide a means for testing for cross-protection between these viruses.

Maramorosch (1956) allowed nymphs of *Macrostes fascifrons* to acquire virus from aster plants with yellows for 2 days. He then anesthetized them, surface-sterilized them, cut each of them into 10 to 12 pieces and placed the pieces in a culture medium containing antibiotics. Virus was recovered from the tissue pieces after maintaining them for 10 days in the solution, but not from the culture fluids, or from similar tissue fragments tested at the beginning of the 10-day period. The experiment provided further evidence for the multiplication of the virus in the tissues of the vector.

Maramorosch (1958b) has demonstrated that if the Rio Grande strain of corn stunt virus is first acquired by the vector *Dalbulus maidis*, it cannot subsequently transmit the Mesa Central strain. However, in the leafhopper, protection in the reverse direction is incomplete and, in plants simultaneously infected with both strains, symptoms of the Mesa Central strain may be overcome and suppressed by those of the Rio Grande strain.

Recently, Littau and Maramorosch (1956, 1958) have reported the first discovery of a cytopathogenic effect of a virus in its leafhopper vector. Such an effect was searched for previously but unsuccessfully by Kunkel (1926), Dobrosky (1931a,b), and Fukushi (1934). Littau and Maramorosch reported that the aster yellows virus caused changes, which are more readily observed in the male than in the female, in the fat body cells of *Macrostelus fascifrons*. In these cells, the cytoplasm becomes sparse, the nuclei become stellate, and the cell membranes become indistinct. This provides evidence against the possible multiplication of aster yellows virus in the presumed symbionts (Black, 1953b, p. 411) which occur in another tissue, the mycetome. Finally, Jensen (1958) demonstrated that the average life of leafhoppers of *Colladonus montanus* that acquired and transmitted peach yellow leaf roll virus was shorter (22 days) following acquisition than that (55 days) of insects that failed to transmit. This finding indicates that this virus multiplies in and is pathogenic to its leafhopper vector.

The cumulative evidence from these many studies now clearly indicates that there are many leafhopper-borne viruses which infect their leafhopper carriers as surely as they infect their plant hosts although in most cases the infection of the leafhopper is inapparent.

#### IV. EVALUATION OF KINDS OF EVIDENCE FOR MULTIPLICATION

The above history of the problem of multiplication of certain viruses in both plants and insects reveals a considerable variety of approaches. It would seem worthwhile at this stage briefly to recapitulate and reconsider these techniques, keeping in mind their possible application to other viruses. Some of the techniques have been enormously expensive in both time and effort; it would be an advantage to use the less costly approaches if they are adequate. It should be remembered that all techniques must ensure that any increase in virus that is demonstrated cannot be due to fresh acquisition of virus from plants.

There are two techniques which have been used, which admit of no other interpretation than multiplication in the vector. They are:

(1) Serial passage of virus from insect to insect by injection technique until the dilution attained exceeds with certainty the maximum dilution of the starting material that can be successfully inoculated. This technique



involves less work than the following one and, when it is available, it would seem to be preferable.

(2) Transovarial passage of virus from generation to generation until the dilution attained exceeds with certainty the maximum possible without multiplication in the insect. Black's estimate (1950) that a dilution of  $10^{-12}$  should be exceeded still seems to be of the correct order of magnitude.

In addition to these two unequivocal methods there are others that can provide evidence indicating with high probability that multiplication occurs. These are:

(1) Methods of titrating for virus increase in the insect. Demonstrated increases are almost certainly due to multiplication, but this evidence is subject to the possibility that a given quantity of virus may, for some reason, be detectable at a higher dilution at one time than another (Black, 1941).

(2) Cross-protection between strains of virus in the vector. This phenomenon would seem to depend almost certainly upon multiplication in the vector. However, interference between two strains of a plant virus at time of entrance into the host was demonstrated by the work of Beale (1947) and would seem to be a remote alternative possibility to interference through multiplication.

(3) A cytopathogenic effect in the vector. Symptoms of disease in the cells of the insect almost certainly indicate that virus is multiplying in those cells. However, a toxic effect without multiplication would seem to be a remote possibility. The same considerations also apply to other symptoms of disease.

(4) Persistence of virus in the vector and frequency of transmission by single insects (Kunkel, 1954), when independent of or not proportional to the length of the acquisition feeding period, have been associated with multiplication in the vector and stand in marked contrast to the contrary condition in curly top, where the evidence is against multiplication.

In addition, there are a number of other kinds of evidence for which multiplication in the vector is the most probable basis, but which are also subject to other interpretations.

There is abundant evidence to support Kunkel's interpretation of his heat-induced incubation periods in the vector of aster yellows virus, but the phenomenon by itself, without evidence from other kinds of experiments has been variously interpreted.

Black (1954, p. 84) has emphasized the long incubation periods associated with all those viruses that have been demonstrated to multiply in the vector. However, Kunkel (1948), on the one hand, has correlated both long and short incubation periods in the vector with long and short incubation periods in the plant where multiplication is known to occur and, on the other hand, Smith and Lea (1946) have given hypothetical explanations for both long and short incubation periods, without assuming multiplication in the vector.



Maramorosch (1950a,b, 1953b) has demonstrated that the greater the inoculum the shorter the minimum incubation period following injection, and has considered this as evidence for multiplication. However, even if there were no multiplication, but mere internal passage, one would expect that the greater the quantity of inoculum injected the shorter the minimum incubation period that one could detect. Similarly, low temperature might be expected to lengthen the incubation period or even arrest transmission whether or not multiplication occurred (Maramorosch, 1950a, 1953b).

Day (1955) and Heinze (1956) have succeeded in detecting potato leaf roll virus in solutions. This was accomplished by the difficult operation of injecting virus in solution into aphids. Aphids had not been successfully inoculated by injection before. Both authors interpreted their results in obtaining successful inoculations as evidence for multiplication in the vector. However, there would seem to be no inherent reason why injections should not be successful with a virus should it have the property of passing through the tissues of the vector without multiplication. In preliminary experiments, Maramorosch (personal communication) has been unable to transmit the beet curly top virus serially in the vector, in spite of his successful transmission of the virus to the vector by injection.

The viruses of rice stunt, clover club leaf, wound tumor and rice stripe are known to pass transovarially and to multiply in the vector, but there would appear to be no *a priori* reason why a virus which does not multiply in its vector but passes through vector tissues could not be transmitted transovarially.

## V. EVALUATION OF KINDS OF EVIDENCE FOR ABSENCE OF MULTIPLICATION

If any plant virus passes internally through the vector without multiplication, a demonstration of the fact is inherently much more difficult than a demonstration of multiplication. In fact, with our present degree of refinement in virological techniques, it does not seem possible to provide rigorous proof of such a relationship. Nevertheless, a good deal of evidence has been obtained indicating that the leafhopper-borne curly top virus does not multiply in its vector although it is retained through the molt and is capable of being transmitted for days after a single acquisition feeding.

The data against multiplication vary in significance. The most critical would seem to be:

(1) The demonstration that the virus content decreases progressively and consistently following termination of the acquisition feeding period. By feeding extracts of leafhoppers to non-viruliferous leafhoppers and testing the latter on beets, Bennett and Wallace (1938) demonstrated such a decrease

for curly top virus. By injecting extracts from aphids into virus-free aphids which were later tested on plants, Harrison (1958a) demonstrated such decrease for potato leaf roll virus. In the propagative viruses, aster yellows and wound tumor, measurements indicate an increase in virus after acquisition (Black, 1941; Maramorosch, 1956). The progressive decrease in virus content following acquisition would seem to be the most significant datum that can be obtained as evidence against multiplication. However, it serves to illustrate the difficulty of attempting to prove this negative proposition. Apart from the purely theoretical impossibility of proving the complete absence of multiplication, there is the practical obstacle of being unable, at present, to quantify these viruses accurately or to know how many virus particles are present in an infective dose. Thus, it is theoretically possible that, with the techniques now available, a progressive and consistent decrease in the activity of a massive dose of acquired virus could be measured while, at the same time, the multiplication of some virus might occur below the level of the concentration that can be measured.

(2) The demonstration that transmission by single vectors consistently decreases following the termination of certain appropriate acquisition feeding periods. This was proved for curly top virus by Freitag (1936) and by Bennett and Wallace (1938). Bennett and Wallace found that this decrease was not very noticeable when the acquisition feeding period was such that the original virus content was high. A minimum latent period of 4 hours, during which the insect is unable to transmit, is detectable in suitably designed experiments with curly top. Therefore, a period of maximum transmission, at some point between the latent period and the period of decreasing transmission, is to be expected. The possibility that this decrease in transmissive ability may be due to aging of the leafhoppers was thoroughly investigated in the case of curly top virus. Freitag (1936) showed that the percentage of leafhoppers able to acquire and transmit virus decreased with age. Nevertheless, age was not the only factor involved, because transmissive ability could be increased by a second acquisition feeding (Freitag, 1936; Bennett and Wallace, 1938). Giddings (1950) showed that individual leafhoppers transmitting curly top virus, strain 2, could transmit the strain 3 virus following a 16-hour acquisition feeding period. The proportion of transmissions of strain 3 virus rose to 50 % and subsequently fell to 9 %. A second acquisition feeding period on a source of strain 3 increased the proportion of strain 3 transmissions. This effectively ruled out age as a sufficient explanation for decrease in transmissive ability with time.

(3) The demonstration that transmissive ability of *individual* leafhoppers is roughly proportional to the length of the acquisition feeding period. Bennett and Wallace (1938) showed this to be true for the curly top virus when they found that although virus could be acquired in as short a time as

1 minute, it required an acquisition feeding period of 2 days for individual leafhoppers to achieve their maximum ability to transmit. This result is in striking contrast to the results of Kunkel (1954) with aster yellows.

Other kinds of data would appear to be of less certain significance. For curly top and potato leaf roll, the virus content of the vectors was shown to increase with increase in the length of the acquisition feeding period (Bennett and Wallace, 1938; Harrison, 1958a). However, these measurements were made on colonies of vectors. It is the obvious expected relationship if no multiplication occurs. However, it should also be true in cases where multiplication is known to occur and where the proportion of infective insects in the colony increases in proportion to the length of the acquisition feeding period, as in aster yellows (Kunkel, 1954). The criterion would be significant if it were applied to individual insects, except that, conceivably, it might not distinguish between no multiplication and limited multiplication in local lesions.

Giddings (1950) obtained abundant evidence that strain 2 of curly top virus did not prevent acquisition and transmission of strain 3. The reverse order was not as thoroughly tested because the opportunity to acquire strain 2 followed so shortly after the acquisition of strain 3, but the results gave no indication of protection. Harrison (1958b) has demonstrated that acquisition of a mild strain of potato leaf roll virus does not prevent subsequent acquisition and transmission of a severe strain of the same virus by the aphid vector.

It is important that the cross-protection test between two viruses in the vectors be carried out in both directions. Maramorosch (1958b) has shown that when the Rio Grande strain of corn stunt virus is acquired first by the vector it is unable to acquire and transmit the Mesa Central strain two weeks later. However, if the strains are offered in the reverse order, the Mesa Central strain is transmitted first and the Rio Grande strain later. Protection was afforded in the one sequence but not in the other, in spite of the fact that Maramorosch's (1952b) results on serial passage of this virus from vector to vector indicated that it is propagative. Chamberlain (1958) has reported lack of cross-protection in the mosquito between eastern and western equine encephalomyelitis viruses. These viruses are serologically related (Casals, 1957) and multiply in the mosquito. Experiments with various viruses and vectors to date indicate that interference between viruses in the vector may signify virus multiplication in the vector. However, it seems clear that lack of interference does not necessarily indicate the absence of such multiplication.

It is noteworthy that Harrison (1958a,b), as mentioned above, obtained three kinds of evidence in harmony with the hypothesis that potato leaf roll virus does not multiply in the aphid vector but that D. Stegwee and M. B. Ponsen (in work to be published) have obtained evidence that this virus

multiplies in its aphid vector. By using a refined injection technique they obtained transmission of potato leaf roll virus from aphid to aphid through 15 serial passages while maintaining them on Chinese cabbage which is immune to the virus. Their measurements indicated that the virus was diluted 1/20 at each passage. Nonviruliferous aphids failed to recover virus from the Chinese cabbage plants on which the injected insects were maintained. These results demonstrated infectivity in the terminal passages, at dilutions far in excess of those possible with the starting inoculum. The results of Stegwee and Ponsen suggest that even the evidence for progressive decrease of virus content in vectors following termination of acquisition feeding, for increase of virus content in proportion to length of acquisition feeding period and lack of interference between virus strains in the vector, may have to be interpreted with even more caution than has been true in the past.

Certain data that have been advanced as evidence against multiplication are known to be unreliable. Black (1953b, p. 411) has pointed out that certain criteria, such as loss of infective ability (Kunkel, 1926), irregularity of transmission, or even failure to transmit during the whole life (Fukushi, 1940) or for many weeks (Black, 1950) in the life of a virus-bearing insect cannot be considered significant evidence for absence of multiplication, because these phenomena are exhibited by some viruses that have been proven to multiply in their vectors. Loss of virus followed by its reacquisition does not, of itself, eliminate multiplication, because this may happen in both plant and animal virus infections.

Although the minimum noninfective period in the leafhopper following acquisition of curly top virus is not so short that virus multiplication could not have occurred, it is nevertheless in striking contrast to the minimum incubation periods of about a week or more in all cases where multiplication in the vector has been demonstrated. However, whether short and long incubation periods are indicative of the absence and presence of multiplication, respectively, as suggested by Black (1954), cannot be determined until the accumulation of critical evidence on more cases.

None of the critical tests applied to curly top virus has provided evidence for multiplication in the vector. This indicates that certain properties that at times have been attributed to multiplication can no longer be necessarily so assessed.

One of these properties is a high degree of vector specificity. In the case of curly top, this specificity has recently played a dramatic role in revealing the probable past history of the virus and vector. Only a single vector, *Circulifer tenellus*, for the curly top virus has been found in North America. Oman (1948) pointed out that *C. tenellus* had close relatives, not in the United States, but in the Mediterranean area. Leafhoppers apparently identical to *C. tenellus* were found in North Africa, South Africa, Sicily, and Palestine (Frazier, 1953;



Young and Frazier, 1954). *C. tenellus* from North Africa were shown to interbreed with *C. tenellus* from the United States and to transmit the North American virus (Freitag *et al.*, 1955); finally, the curly top virus was found in Turkey and shown to be transmissible by *C. tenellus* of the United States (Bennett and Tanrisever, 1957). These findings support the hypothesis that both the curly top virus and the vector were introduced into North America from the Mediterranean region.

The specific relations of this circulative virus are emphasized by the demonstrated vector specificities of varieties of the virus. The variety *distans* of Argentina is not transmitted by *C. tenellus* but by *Agalliana ensigera* (Bennett *et al.*, 1946); the variety *brasiliensis* from Brazil is not transmitted by *Agalliana ensigera* but by *Agallia albidula* (Bennett and Costa, 1949); and another Brazilian variety, *solanacearum*, is not transmitted by *Agallia albidula* but by *Agalliana ensigera* and *Agallia sticticollis* (Costa, 1952). All these findings indicate that a high degree of specificity may be found in circulative viruses.

The curly top virus studies also revealed genetic differences in transmissible ability of individual insects in *C. tenellus* (Bennett and Wallace, 1938). In other words, even if the virus does not multiply in the vector, the frequency with which individual infective insects transmit the virus and the proportion of insects that become infective under given conditions is genetically determined.

## VI. SIGNIFICANCE OF BIOLOGICAL CYCLES

Kunkel (1926) considered that the incubation period of the propagative aster yellows virus in the vector is a period of multiplication. Titration of the virus content during the incubation period provides direct evidence that this is so (Black, 1941; Maramorosch, 1953a). In two cases, Maramorosch (1953b), was able to get transmission of aster yellows virus on the day the vectors were injected, but not on subsequent days. The fact that this occurred only when the maximum possible dose of virus was injected supports the idea, advanced by Kunkel (1937) in his interpretation of his heat-induced incubation periods, that the virus must reach a certain concentration before transmission can occur. Multiplication during the incubation period is supported by the evidence that the aster yellows virus could be detected at low concentration at the fourth day of an 18-day incubation period but increased at least 100-fold between the beginning and end of the incubation period (Black, 1941). Maramorosch's results (1953a, 1956) support these findings.

Maramorosch (1953a,b) considered that the failure to detect virus by the insect injection technique during the first few days of the incubation period might indicate the existence of an eclipse period similar to that occurring during phage reproduction. This would imply that the virus exists in different



developmental stages in the vector. He believed that because of the sensitivity of the method the eclipse period appeared to be real and that there was evidence for the existence of two developmental stages, infective (mature) and noninfective (vegetative) virus. The only one of the propagative viruses for which it is possible, at present, to estimate the sensitivity of the insect injection method is the wound tumor virus. It has already been estimated that the lowest concentration of particles of this virus that will react serologically is about  $10^9$  per milliliter and that this represents a virus preparation from insects diluted to  $10^{-2.5}$ , when the concentration in insects themselves is taken as unity. The end point for infectivity in insect injection experiments is greater. One infection has been obtained at a dilution of  $10^{-5}$  and 8 at  $10^{-4}$ . Since the volume of solution injected into insects averaged about  $0.1 \mu\text{l}$ . (Black and Brakke, 1952), this would indicate that infection was obtained, in the one case, with about  $10^{2.5}$  virus particles and, in the other cases, with about  $10^{3.5}$ . The former figure is an estimate of the minimum number of wound tumor virus particles necessary for successful inoculation of the vector by injection. The data indicate, however, that successful inoculation with  $10^{2.5}$  particles is only attained if many trials are made; it would be safer to consider  $10^{3.5}$  particles as a practical minimum number with present techniques. These minimum numbers are much lower than those for any other infectivity assay of virus that infects plants. Nevertheless, the calculation indicates that the insect injection technique is not sensitive enough to ascertain whether or not an eclipse period really exists—a possibility which was considered in Maramorosch's (1953a,b) discussions. It indicates, instead, that a considerable increase in fully formed infective virus occurs during that part of the incubation period that has been suggested as the eclipse period, before the virus concentration reaches a level great enough to be detected. This does not mean there is not an eclipse period during the cycle of propagative viruses in insects; it does indicate that, at present, we have no direct evidence for such an eclipse period. What evidence there is on the reproduction of other viruses would suggest that an eclipse period is a part of the virus cycle of reproduction in the plant and insect, before and after the end of the incubation period. The presence of soluble viral antigen of wound tumor in both insect and plant supports this probability.

In speculating upon the possible reasons for the high mortality in the second passage of virus in series through the vector, Maramorosch (1953a,b) suggested that the aster yellows virus, in its behaviour toward the vector, might be comparable to a lysogenic phage in its relationship to the bacterial host. However, except for a few days at the beginning of the incubation period, the insect is the best source of infective virus of which we know. This is the opposite of the lysogenic relationship. The recent finding of a cytopathogenic effect in the vector itself is also contrary to the lysogenic relationship.

Kunkel (1938, p. 22) considered multiplication as the basis for the specific relationship that exists between the aster leafhopper and the aster yellows virus. The specificities in the relationships of leafhoppers and their viruses have been discussed earlier (Black, 1953c). Since then an even more specific relationship has been described by Maramorosch (1958a), who found that *Macrostelus laevis*, the vector of European aster yellows virus, would not transmit strains of aster yellows virus of either the eastern United States or California, although these were transmitted by *M. fascifrons*. Work by Maramorosch (1952b) has a bearing on the question of multiplication being the basis for specificity. Preliminary evidence was obtained suggesting that corn stunt virus could be transferred serially from vector to vector. Maramorosch also obtained evidence that this virus and aster yellows virus were able to maintain themselves, for at least 19 days, in certain nonvector species of leafhoppers that had acquired them by feeding on diseased plants. The fact that he could not detect virus in the nonvectors 2 days after acquisition, but could detect it 19 days after acquisition, may mean that some multiplication of virus occurred in the nonvector species. However, attempts to recover the two viruses after three blind serial passages in nonvector leafhoppers failed, a result which indicated no multiplication or limited multiplication in the nonvectors. If multiplication does occur in nonvectors, then the concept that multiplication determines specificity of transmission in these viruses must undergo some modification. Also, curly top virus, for which the weight of evidence appears to be against multiplication, is one of the most specific in its vector relationships.

Black (1941, p. 126) discussed the implications deriving from any demonstration that a plant virus could multiply in its insect vector. He considered that it would provide strong evidence for the relationship of plant and animal viruses and would serve as a connecting link between the two groups. In fact, he considered that a virus multiplying in both insects and plants was really both a plant and animal virus. He also reasoned that it did not support any precursor hypothesis of virus multiplication which required, in any medium supporting virus replication, the existence of an element of approximately viral size, which, on undergoing a change involving a comparatively small fraction of its total structure, became the active virus. He thought that virus growth might be akin to the assimilation of simple compounds by organisms.

Kunkel (1941, p. 769) considered that aster yellows virus must be classified as an obligate heteroecious parasite, like the plant rusts, in that it must alternate between widely different hosts. In the case of this virus the obligatory alternation in nature is between an insect and a plant species.

Black (1953b) emphasized the bearing of these viruses upon virus classification, expressing the opinion that the current primary divisions of bacterial,

plant, and animal viruses would have to be replaced eventually by some more basic classification.

Bawden (Black, 1953b, p. 413) considered that the demonstration of virus multiplication in both plants and insects favored their symptomless insect vectors as their original evolutionary source. This concept has been expanded by Maramorosch (1955). He pointed out that the insect vector host range is narrow, comparatively speaking, and the plant host range usually quite broad and believed that this, as well as the lack of symptoms in the



FIG. 1. Schematic representation of the life cycle of wound tumor virus. Clear drawings represent virus-free hosts; stippled drawings, hosts in which virus increase is occurring; black drawings, fully infected hosts. The right half of the circle represents virus propagation in the plant; the left half, virus propagation in the insect vector. The latter phase may be repeated directly by transovarian passage of virus. (Modified from Black, L. M., 1951, in *Brooklyn Botanic Garden Ann. Rept. for 1950-1951*: 32-33. Drawing by Natalie H. Davis.)

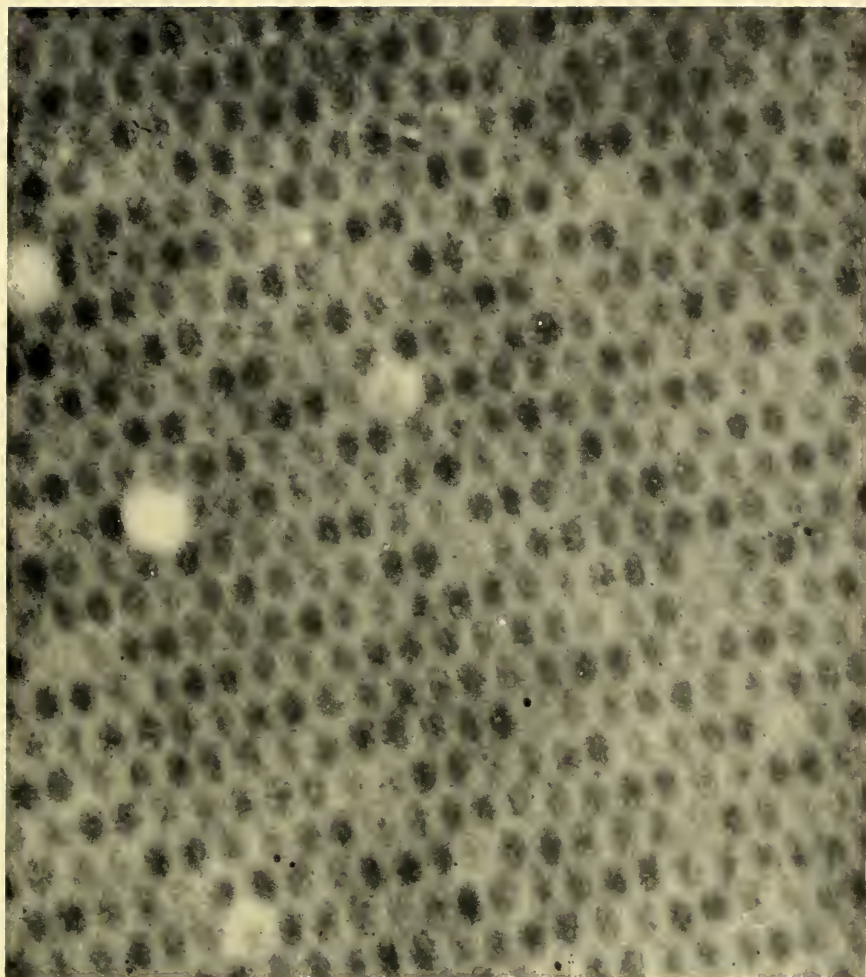


FIG. 2. Electron micrograph of a section through a crystal of purified wound tumor virus (Magnification  $\times 110,000$ ). (Courtesy Vatter, A. E. and Whitcomb, R. F.)





vector, indicated long adaptation to the arthropod host. However, it is one of the small ironies of scientific investigation that Littau and Maramorosch (1958b) have provided the first evidence against this hypothesis by finding a pathogenic action of the virus on the arthropod, an action that is quite drastic in some affected cells. Maramorosch (1955, p. 246) considered that the relationships of these viruses and what we know of their morphology seemed to place them in the category of microorganisms.

Black (1953b) has favored the hypothesis that these viruses have evolved by retrograde evolution from organisms in the manner first proposed for all viruses by Green (1935). Green's theory conceived of the progressive loss by parasitic organisms of physiological functions that could be provided by the host. He postulated that this loss of function would result in concomitant loss, simplification, and reduction in structure. Evolution along such lines might ultimately give rise to agents able to reproduce themselves at a molecular level at which only the most essential components for reproduction were retained. This theory appeals to the author because loss of physiological function, and morphological simplification and reduction are so common in parasitism as to suggest that in time they are inevitable. Also, in respect to size, a very complete gradient of parasitic agents is known. Viruses consist of nucleoproteins which may be the residuum of reproductive material one would expect to persist in the ultimate products of such evolution. Green's theory has withstood the test of more than twenty years of crucial advance that has been revolutionary to our understanding of viruses. In the light of this theory, an attractive hypothesis for the origin of the viruses with reproductive cycles in plants and insects (Fig. 1) would postulate their origin from organisms which at one time were saprophytic or commensal in both plant and insect. From this state, forms may have emerged that were parasitic in both, but not obliged to alternate between them. Obligatory alternation of hosts might then have arisen as a possible advantage or refinement for parasitism. Once an organism had evolved to the stage in parasitism where the alternation of hosts was obligate, reduction to the virus level may have occurred. Among the viruses proven to multiply in both plants and insects, wound tumor virus (Fig. 2) is the only one whose morphology is known. The long incubation periods and persistence in the vector of potato yellow dwarf and tomato spotted wilt viruses suggest that they may multiply in their vectors. The morphology of these viruses is also known. About others in the group, or suspected to be in the group, we know nothing certain regarding morphology. It would seem to be significant that all three of these viruses are larger than any other plant virus the morphology of which is known. The alternate cycle in such widely different hosts might have reduced the number of dispensable physiological functions and this in turn may have limited the reduction in size.

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## Chapter V

### Bacteriophage as a Model of Host-Virus Relationship

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#### I. INTRODUCTION

Between 1860 and 1915, numerous bacteriologists had handled a considerable number of bacterial cultures. Many of them had certainly observed the disappearance of bacterial population or colonies. Among them was d'Hérelle, who, around 1910, described the "taches vierges" of the colonies of *Coccobacillus acridiorum*. A few years later, Twort observed the vitreous degeneration of some colonies of a *Micrococcus*. He had, however, the idea that bacterial lysis could be an "acute infectious disease." He succeeded in transferring the active principle from vitreous to normal colonies, showed that it was filterable, that it multiplied and reproduced true to type, and that the number of lesions was inversely proportional to the dilution. In his classic paper, which appeared in 1915, Twort discussed the possible nature of the infectious agent: it could be an ultravirus, or a small parasite reproducing at the expense of the bacterium, or a phase of the life cycle of the micrococcus, or an autocatalytic enzyme, or a primitive form of life.

The existence of acute infectious diseases of bacteria was confirmed two years later by d'Hérelle, who named the agent *bacteriophage*. This was the origin of extensive investigations. Bacteriophage is now the virus about which we possess the most extensive information, and is an excellent model for the understanding of viruses.

Until the end of the nineteenth century, the history of viral diseases is just a part of the history of infectious diseases. In about 2500 B.C., the Chinese



had identified smallpox and knew that it was transmissible. Aristotle was aware of the fact that rabies was transmitted by the bite of dogs; the Hebrews used to compare this bite to that of a venomous snake. In Latin, *virus* means "venom" or similar poisonous fluid. Virus was something which could produce a disease. And in A.D. 50, Cornelius Aulus Celsus produced this remarkable sentence: "Rabies is caused by virus."

Ideas concerning infectious diseases remained metaphysical until the notion of a specific agent emerged, and until, mainly as a consequence of Pasteur's work, the agents of infectious diseases were identified as microbes. These agents, whether bacteria, protozoa, or fungi, were called viruses.

Pasteur *et al.* (1884) had no difficulty in proving that rabies was a specific infectious disease. Although they were unable to see the agent, they quite naturally considered it a small microbe. When Iwanowsky (1892) discovered that the juice of tobacco plants showing the symptoms of mosaic remained infectious after filtration, he also concluded that the infectious agent was a small microbe. Then came Beijerinck, who confirmed the filterability of tobacco mosaic virus. He also discovered that the infectious power was not lost by precipitation with alcohol and that the infectious agents could diffuse through agar gels. The infection, wrote Beijerinck, is not caused by microbes but by a fluid infectious principle. This intuition of a difference of *nature* between tobacco mosaic virus and microorganisms makes Beijerinck the real founder of conceptual virology, that is to say, of virology.

Beijerinck's views were so opposed to the current ideas that they did not receive any attention. This did not prevent the discovery of a number of filterable agents which were considered to be small infectious agents, in other words, small microbes and which were therefore called filterable viruses or ultra-viruses. Then someone remarked that, because ultraviruses were small microbes, they should be called inframicrobes. For obvious reasons, none of the scientists studying filterable infectious agents was pretentious or modest enough to describe himself as an ultravirologist or as an inframicrobiologist. And as everybody has to be labelled, these scientists were labeled as virologists. Quite naturally, the ultraviruses, the filterable invisible infectious agents studied by virologists, became viruses. And, as a counterstroke, the microbes, the visible infectious agents, were deprived of their ancestral right to be called viruses.

Today, most scientists agree to subdivide the infectious agents into two groups: microbes—including bacteria, algae, protozoa, fungi—and viruses. What is a virus? In order to answer this question, we shall consider bacteriophage as the model of a typical virus, and compare it with typical microorganisms and with typical cellular organelles. We will have to find out what the discriminative characters are which allow us to separate viruses from microorganisms and from organelles. This will lead us to discuss the notion of

infectivity and of pathogenicity. Some virologists consider viruses as organisms, others, as cellular organelles, others, as molecules. This diversity exemplifies the difficulty of drawing a border line between viruses and other entities. The task is hard, but has to be performed if we want to gain a clear picture of the virus.

## II. THE BACTERIOPHAGE

A bacteriophage is a particle built up of nucleic acid and protein and provided with a tail. When the tip of the tail meets a receptive bacterium, it attaches itself to the bacterial wall. Then the genetic material of the phage, mostly nucleic acid, passes into the bacterial cytoplasm. The infected bacterium can evolve in two ways: (1) The vegetative phase is initiated, bacteriophage particles are produced, and the bacterium dies. (2) The genetic material does not enter the vegetative phase. It is converted into a prophage. The bacterium remains alive and is now lysogenic. Let us consider more closely these two types of bacterial responses (Fig. 1).

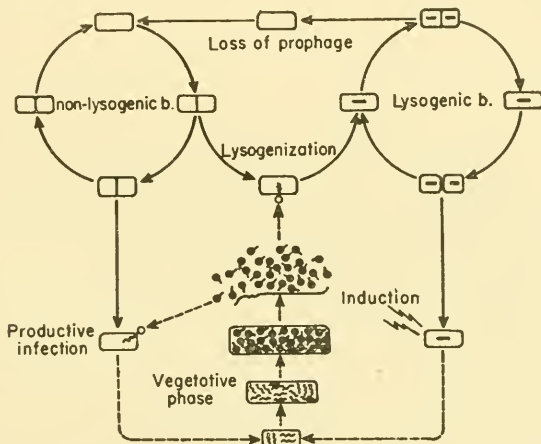


FIG. 1. The life cycle of a temperate bacteriophage. The prophage is represented by a dash; b = bacterium.

### A. The Vegetative Phase

The vegetative phase is characterized by the multiplication of the genetic material of the phage, by the synthesis of phage proteins, and by the morphogenesis of phage particles. Each infected bacterium produces some 100 particles which are liberated as a result of bacterial lysis.

How is phage reproduced during the vegetative phase? It is admitted that the phage constituents, the amino acids, the purine and pyrimidine bases, and

the sugars, are synthesized by the enzymatic machinery of the bacterium. It is admitted also that the bacterium provided the Lipmann system, that is to say, the system of enzymes which converts the potential energy of foodstuffs into the high energy bonds which are necessary for syntheses and the production of a specific pattern. This does not mean at all that bacteriophage is completely devoid of enzymatic activity. When a bacteriophage particle attaches itself to a bacterium, the bacterial wall is altered by an enzyme of the phage tail. Very early during phage development, the bacterial nucleus is destroyed; it is possible that this is due to another phage enzyme. The current view is that during its vegetative phase the bacteriophage diverts a part or the totality of the essential metabolites synthesized by the bacterium and turns them into phage material: nucleic acids and proteins. It acts as a parasite and it is a strict parasite.

It is important to remember that the bacteriophage particle never is reproduced as such: it neither grows nor divides. An infectious particle is never the direct descendant of another infectious particle. It is produced *de novo* by the organization of phage material, proteins, and nucleic acids. And it is also essential to know that the vegetative phase of the cycle has its origin in the genetic material of the bacteriophage.

### *B. The Proviral Phase and the Lysogenic Bacterium*

The genetic material of the phage does not multiply. It reaches a specific locus or receptor of the bacterial chromosome and attaches itself to it. By virtue of this attachment, the properties of the genetic material of the phage are modified and it is then called a prophage. The prophage behaves as if it were a bacterial gene. It divides, together with the bacterial chromosome and, at bacterial division, is transmitted to each of the daughter bacteria. Bacteria which carry a prophage are called lysogenic. They are endowed with the capacity to produce phage in the absence of infection. The prophage is the material base of this property. It is the structure which carries the information necessary for the production of phage particles.

A lysogenic bacterium generally divides without producing bacteriophage particles. The production of infectious entities happens spontaneously, that is to say, without known cause, with a certain probability varying with the systems from  $10^{-2}$  to  $10^{-5}$  in the interval of two divisions. In some lysogenic strains, phage production can be induced at will in the totality of the population by inducing agents, such as UV light, X-rays, organic peroxides, or nitrogen mustard. The current hypothesis is that inducing agents act by modifying the nucleic acid. As a result, the genetic material of the bacteriophage is detached from the bacterial chromosome, and it ceases to be a prophage. The vegetative phase is initiated, ending in infectious phage particles, and the bacterium is lysed.

When analyzing the various phases of the life cycle (Table I), it seems as though the genetic material, the specific nucleic acid carrying the information, is the only structure which the three phases have in common. The multiplication of the genetic material takes place during two phases: in the lysogenic bacterium and during the vegetative phase. In a lysogenic bacterium, no phage proteins have been so far detected and it would seem that phage proteins are synthesized only during the vegetative phase. The vegetative phase is the only pathogenic phase of the cycle and the phage particle the only infectious phase.

TABLE I

CONSTITUTION AND PROPERTIES OF A BACTERIOPHAGE AT THE VARIOUS PHASES OF ITS LIFE CYCLE

|   | Prophage | Vegetative phage | Phage particle |
|---|----------|------------------|----------------|
| Specific nucleic acid   | +        | +                | +              |
| Replication of nucleic acid   | +        | +                | 0              |
| <div style="display: inline-block; vertical-align: middle;"> <div style="font-size: 3em; vertical-align: middle;">{</div> <div style="display: inline-block; vertical-align: middle;">           Synthesis of proteins<br/>           Pathogenicity<br/>           Morphogenesis of particles         </div> </div> | 0        | +                | 0              |
| Infectivity   | 0        | 0                | +              |

A bacteriophage is obviously the sum of the various phases of its life cycle. It would be arbitrary to decide that supremacy should be given to one phase rather than to another: multiplication of the genetic material, synthesis of proteins, and infection are parts of the life cycle. A bacteriophage is therefore a strict parasite of bacteria possessing an organized infectious phase. It is produced from its genetic material and it is multiplied as genetic material.

### III. THE ORIGIN OF BACTERIOPHAGE

Most of those who have discussed the problem of the origin of bacteriophage have considered the infectious particle. Let us instead consider briefly the problem of the origin of the prophage.

Two main theories have been proposed: (1) the prophage is the residue of the degradation of a parasitic bacterium or of a more or less primitive organism; (2) the prophage was born by genic or chromosomal mutations of a bacterium which thus became lysogenic. Instead of trying to discover which theory is right, let us try to build a third theory which would suppress the opposition.

Bacteria are a very homogeneous and certainly monophyletic group, which differ from all other protista by the organization of the mitochondrial



apparatus. This means that *Escherichia coli*, for example, and the hypothetical parasitic bacterium which is supposed to have degenerated into a prophage have a common ancestor. In other terms, the genetic material of *E. coli* and the genetic material of the prophage have originated from the very same genetic material. And the common structure, which has been preserved in the bacterial chromosome as a receptor, and in the genetic material of the temperate phage where it accounts for lysogenization, is the signature of the common origin.

If one admits that the genetic material of the prophage had its origin in the genetic material of a given bacterium, it could as well have originated in another one and especially, as pointed out by S. E. Luria, in an ancestor of the very bacterium which is now lysogenic. The phase at which evolution has taken place is not as important as the existence of a common origin. The endogenous and exogenous theories can be reconciled in the following formula: the genetic material of the bacteriophage and the genetic material of the bacterium have evolved from a common structure, the genetic material of a primitive bacterium.

Whatever the unknown ways might have been by which a bacteriophage has evolved from something which was not the present-time bacteriophage, it has now reached a certain state; it now exhibits certain features which have to be considered independently of their possible origin.

#### IV. IS BACTERIOPHAGE AN ORGANISM?

Organisms might be visualized as independent units of integrated structures and functions. Is bacteriophage an organism?

Let us examine and compare the bacteriophage on the one hand, and, on the other hand, a typical microorganism, such as protozoon, a yeast, or a bacterium.

1. All typical microorganisms contain both types of nucleic acids, DNA and RNA. Bacteriophage contains only one type.<sup>1</sup>

2. All typical microorganisms are reproduced from the integrated sum of their constitutive parts—nucleus, cytoplasm, cytoplasmic organelles, and cortex. Bacteriophage is produced or reproduced from its genetic material.

3. The essence of the multiplication of any cell or microorganism is the replication of the genetic material, but multiplication is not separable from growth, which is the result of the synthesis of cell material. During the growth of a microorganism, the individuality of the whole is maintained. Growth culminates in binary fission. An individual molecule cannot undergo fission. The replication of nucleic acid by the template mechanism is not a

<sup>1</sup> This might be true only for the proviral and the infective phase, not for the vegetative phase.



binary fission.<sup>1</sup> Binary fission has no meaning at the molecular level. It has a meaning only if one considers either a complex organelle or structure or an organism.

The essence of phage multiplication is also the replication of the nucleic acid. During the vegetative phase, there is an increase of phage material but the nucleic acid and the proteins, so far as we know, do not form an organized structure. What increases is a disorderly mixture of phage constituents. There is nothing that could divide. There is no binary fission in a bacteriophage.

4. Microorganisms possess a system of enzymes which convert the potential energy of foodstuffs into the high energy bonds that are needed for biological syntheses, including the production of a specific pattern. This is the "Lipmann system" which is part of the microorganism itself. The Lipmann system is absent in bacteriophage. The energy for the synthesis and production of a specific pattern of phage material is provided by the host cell.

It is manifest that bacteriophage differs from classic microorganism by an impressive number of essential features. A line of demarcation can be easily drawn between bacteriophage, on the one hand, and microorganisms, that is to say, organisms, on the other hand. One could therefore decide that bacteriophage is not an organism. If bacteriophage is not an organism, is it a cellular organelle?

## V. BACTERIOPHAGE AND CELLULAR ORGANELLES

A cellular organelle, or simply an organelle, is a dependent part of an organism. The synthesis of the constituents of an organelle—its growth, its replication, its activity—are controlled by the organism as a whole and in turn the organelle, by the products of its metabolism, takes part in the metabolism of the organism.

In a lysogenic bacterium, the prophage behaves as if it were a bacterial gene. Its replication is controlled by the bacterium as a whole and the prophage is known to modify specifically the bacterium. For all practical purposes the prophage behaves as a cellular organelle. It is a dependent part of an organism, and this organism is not the bacteriophage, but the bacterium. Do we have the right to decide that bacteriophage is a cellular organelle? The fact that bacteriophage, at certain phases of its cycle, behaves as a

<sup>1</sup> It is possible that the Watson-Crick double helix of DNA the duplex, functions as a unit in synthetic processes. If so, from a functional point of view, it is a molecule. The duplex nevertheless replicates. In one of the models proposed for its replication, the two members of the pair separate and each one reproduces its "opposite." The unit of information, the ultimate unit from which a double helix is produced, is the individual single helix which does not undergo fission.

bacterial component is important; however, it appears immediately that bacteriophage is not an ordinary bacterial component. Our task is to find out whether it is possible to discriminate between bacteriophage and typical organelles. Two main characters have to be considered, namely, pathogenicity and infectivity.

Pathogenicity, the power to elicit a disease, is not a characteristic trait of bacteriophage. Bacteria may possess lethal genes which are the potential agents of fatal diseases. Pathogenicity is therefore not a character allowing one to discriminate between bacteriophage and cellular organelles. Let us now consider infectivity.

Infectivity is generally defined as "having the quality or power of communicating disease by infection" and infection is generally defined as "the communication of a disease." Infection and disease are in fact often associated but this is not always the case. When a bacteriophage injects its genetic material into a bacterium, the bacterium may become lysogenic. It remains healthy and multiplies. It is nevertheless clear that the bacterium has been "infected." Infection in this case is *the introduction into an organism of an entity able to reproduce true to type*. According to this definition, the transforming principle should be considered as infectious. The introduction of pathogenicity in the definition of infection would not solve our problem, for one can conceive of a lethal gene introduced in lieu of "normal" transforming principle and producing a disease. Yet everyone has the feeling that the introduction from outside of a lethal gene is something radically different from the introduction of the genetic material of a bacteriophage. We need to find out how transformation can be excluded from infectious processes.

During the vegetative phase of the life cycle of a bacteriophage, organized infectious particles containing proteins and nucleic acid are produced. The differences between a disease started by a lethal gene and a disease started by the genetic material of a bacteriophage are manifold.

1. So far as we know, the lethal gene is not reproduced during the disease, which consists mainly in a disturbed metabolic process that might be the synthesis of an abnormal structure. It is not accompanied by the multiplication of the lethal gene. During the vegetative phase of the phage cycle, abnormal structures such as proteins are also produced but the genetic material of the phage is multiplied.

2. No infective particles are produced during the disease initiated by a lethal gene, whereas infective particles are formed during the vegetative phase of the cycle of a bacteriophage. These are "organized" particles, composed essentially of a nucleic acid and proteins, possessing a definite size and structure.

From all we know concerning the life cycle of a virus, infectivity of a virus is the ability to introduce its genetic material into a cell. Accordingly, an

infection would be the introduction into a cell or an organism of an entity able to multiply, able to produce a disease, and able to reproduce organized infective entities containing proteins and nucleic acid. This definition excludes from infectiousness the transforming principles and the lethal genes which could be introduced from outside.

Bacteriophage differs from cellular organelles by possessing an infectious phase. Table II clearly indicates that bacteriophage differs by essential

TABLE II  
ORGANISMS, VIRUSES, AND CELLULAR CONSTITUENTS

|   | Organisms | Viruses | Cellular constituents |  |
|---|-----------|---------|-----------------------|--|
|   |           |         | Genetic material      | Organelles endowed with genetic continuity |
| Types of nucleic acid   | II        | I       | I                     | ?  |
| Multiplying as nucleic acid and produced from nucleic acid only | 0         | +       | +                     | 0  |
| Growth and division <sup>a</sup>                                | +         | 0       | 0                     | +  |
| Presence of a Lipmann system                                    | +         | 0       | 0                     | 0(+) <sup>b</sup>                          |
| Infectivity   | +         | +       | 0                     | 0  |

<sup>a</sup> Concerns microorganisms only. The replication of a structure by the template mechanism is considered neither as growth nor as division (see p. 192).

<sup>b</sup> Some organelles only contain a Lipmann system.

features from organelles as well as from organisms and that it is neither an organelle nor an organism. A bacteriophage is a bacteriophage. What is bacteriophage?

## VI. BACTERIOPHAGE AS A VIRUS

Bacteriophage, being the small and filterable agent of an infectious disease, is classified among viruses. Why? Obviously, because bacteriophage and viruses have a certain number of features in common. Viruses in general are opposed to bacteria, protozoa, fungi, algae, and more generally to microorganisms. This implies the existence of a category of infectious agents, viruses, which are different from other infectious agents. Viruses are often opposed to microorganisms because of their size. A virus, according to some virologists, should have at least one dimension lower than 200  $m\mu$ . If dimensions have any meaning, it is not by the astrological virtue of a number, but because of a correlation between size and some essential properties which are

responsible for fundamental differences. It happens that a number of small filterable, infectious agents of plant and animal diseases share with bacteriophage those essential features which allow one to separate bacteriophage from organisms and organelles.

These infectious agents can be united into a special category for which the following definition is proposed: strictly intracellular and potentially pathogenic entities with an infectious phase: (a) possessing only one type of nucleic acid; (b) reproduced from their genetic material and multiplied in the form of their genetic material; (c) unable to grow and to undergo binary fission; and (d) devoid of a Lipmann system. It should be noted that any one of the characters (a), (b), (c), (d) is sufficient to identify an infectious entity as a virus and that these characters are probably correlated and subordinated.

The definition of viruses which has been proposed applies to any of the phases of the life cycle—proviral, vegetative, infectious. If one wants to put emphasis on the infectious particle, the following definition can be proposed: viruses are infectious and potentially pathogenic entities, reproduced from their genetic material and multiplied as genetic material, unable to grow and to undergo binary fission and devoid of a Lipmann system.

The problem of the origin of viruses is not solved. Whatever their ancestor might have been, viruses *now* differ from organisms as well as from cellular organelles.

An object, a category, or a concept has an individuality or a reality only because of the existence of different objects, categories, or concepts. The art of definition is founded on differences as well as on resemblances. A definition has to exclude as well as to reassemble. Our definition of viruses is valid only because, at the same time, it includes a homogeneous class of entities, viruses, and excludes other homogeneous classes of entities, such as microorganisms and cellular organelles. Therefore, we do have the right to unite viruses into a special class of objects. The concept of virus has thus been established on a firm ground and the term "virus" has, at last, a definite meaning.

Our position might appear somewhat too dogmatic. One can, of course, point out that between "small" viruses and large microorganisms, one finds intermediary classes of large viruses and of small microorganisms. Taking advantage of this situation, one can decide, as some people do, that viruses are small microorganisms and that microorganisms are large viruses. This is all right, but why then should some infectious agents be called viruses? Because obviously they differ from bacteria, algae, fungi, and protozoa, and because they differ from other microorganisms. If one tries to find out the nature of the difference, one necessarily lands where we have landed. Either viruses do not exist and we should stop utilizing the term "virus," or viruses do exist and we have to state why something is a virus.



Some virologists may find it difficult to decide whether a middle-sized agent is a bacterium or a virus, but if they are willing to look carefully at the thing in question for the characteristic features of viruses the decision should always be possible. As already mentioned we do not know what the ancestors of viruses have been and it remains possible that some have evolved from cellular constituents, other from parasitic microorganisms. A theoretical possibility has therefore to be considered: it may turn out that an infectious entity will sometime be discovered possessing the features of a virus during one phase of its life cycle, for example, multiplication in the form of its genetic material, and the features of a microorganism during another phase, for example, ability to grow. Such a creature would be highly interesting, but it remains a hypothesis.

For the time being, making use of the criteria which have been proposed, it is possible to decide whether an infectious agent or whether a given particle, is or is not a virus. A virus is neither an organism nor an organelle. A virus is a virus.

## VII. ARE VIRAL DISEASES ALWAYS INFECTIOUS?

The virus has been defined by a few biochemical and physiological features associated with two properties, or potential properties, namely, infectivity and pathogenicity. The very title of this section might therefore seem strange.

Classically, an infectious disease is a disease which is started by an infectious entity and ends with more infectious entities. A bacteriophage infects a bacterium, the bacterium lyses and liberates 100 phage particles: this is a typical infectious disease (see Table III).

TABLE III  
SOME DISEASES OF THE BACTERIAL METABOLISM <sup>a</sup>

|  | Infectivity of the |             | Viral disease | Infectious disease |
|--|--------------------|-------------|---------------|--------------------|
|  | Starter            | End product |               |                    |
| Lethal synthesis of bacteriocins                                 | 0                  | 0           | 0             | 0                  |
| Synthesis of phage material from prophage (defective lysogenics) | 0                  | 0           | ?             | 0                  |
| Synthesis of phage <i>particles</i> from prophage                | 0                  | +           | +             | 0                  |
| Synthesis of phage material after infection (abortive infection) | +                  | 0           | +             | 0                  |
| Synthesis of phage <i>particles</i> after infection              | +                  | +           | +             | +                  |

<sup>a</sup> "Infectious disease" is restricted to those cases where the disease is started by an infectious particle and ends in infectious particles.



Sometimes, however, the infection is "abortive." Phage proteins and nucleic acid are synthesized, but are not organized into infectious particles. The disease has been initiated by an infective viral particle and it is characterized by the production of viral material. It is a viral disease, but it is not infectious *sensu stricto*. When phage is produced from prophage, it is the reverse: the disease has been initiated by a noninfectious particle and ends with infectious viral particles. The disease is again viral, but again it is not a "true" infectious disease.

Some lysogenic bacteria called *defective* perpetuate an abnormal prophage. When *induced*, these lysogenic bacteria synthesize phage material and die, but do not liberate phage particles. The disease is not infectious. To decide that it is or not viral is a matter of taste.

Finally, some bacteria, called bacteriocinogenic, perpetuate a potentially lethal gene. When this gene is expressed, the bacteria start synthesizing bacteriocins and die. The disease is a lethal synthesis which is neither viral nor infectious. The production of bacteriocin is a bacterial disease controlled by a potentially lethal gene.

Prophage is also, in a way, a potential lethal gene, for the lysogenic bacterium dies when prophage expresses its potentialities. As already seen, the originality of the disease is that it ends with organized infectious particles which are viruses. And the originality of lysogenic bacteria is the perpetuation of a structure which *behaves as a bacterial gene* and which *is the genetic material of a virus*.

It is therefore useful to remember that viral diseases, considered at the level of individual cells, are sometimes not infectious, that a virus is not always pathogenic and not always infectious. The attributes of viruses—reproduction, infectivity, pathogenicity—are never expressed at the same time in the same structure.

These essential features are present *potentially* in the genetic material, which is the only essential, nondispensable part of a virus. It contains the information necessary for the synthesis of viral constituents and for the morphogenesis of organized infectious particles. Many virologists are inclined to think that the genetic material preceded the virus in phylogeny.

The virus thus occupies a privileged position at the crossroads of normal and pathological heredity, at the crossroads of diseases of the metabolism and infectious diseases.

### VIII. REMARKS ON THE PATHOGENICITY OF VIRUSES

Infectivity and pathogenicity have been considered as indispensable characters for identifying an infectious agent as a virus. So far as infectivity is concerned, this seems justified, but the inclusion of pathogenicity, even of a potential pathogenicity, as a necessary feature of a virus may be questioned.

During the vegetative phase of a virus, during the rapid synthesis of viral proteins and nucleic acids, a more or less important part of the host's metabolism is diverted toward the synthesis of foreign structures. Whether fatal or not, this spoliation might be considered as a disease. The synthesis of a prophage, however, diverts such a small fraction of the bacterial metabolism that it can hardly be considered as a malady. A prophage is not pathogenic, in the sense that it does not produce a recognizable disease. If the vegetative phase of the life cycle, that is to say the production of bacteriophage particles, were itself non-pathogenic, bacteriophage could be visualized as a part of the life cycle of the bacterium, as a sort of aberrant gamete.

When a process ends with the death of a cell or of an organism, pathogenicity cannot be questioned. It is, however, manifest that any disease might be subclinical, and that the definition of normalcy and health is a most arduous task. The viruses which have been studied so far are agents, or potential agents, of diseases. In order to detect a virus one utilizes, among other characters, its pathogenic activity either on a sensitive organism or on a sensitive cell. An entity completely devoid of recognizable pathogenic activity would be difficult to identify as a virus. And yet, a particle could exist, having the life cycle of a virus, including an organized infectious particle of definite size and structure, and be devoid of pathogenicity.

Moreover, pathogenicity is only a potential feature of viruses. The expression of the pathogenicity of prophage depends on the probability of an unknown internal metabolic process of the lysogenic bacterium. The expression of the pathogenicity of the virus sensitizing *Drosophila* to CO<sub>2</sub> depends on an environmental factor, a high CO<sub>2</sub> tension. In one case, expression corresponds to the transition from prophage to the vegetative phase of the cycle. In the other, it is only the revealing of a certain state of the infected *Drosophila*. The probability for a virus to express its potential pathogenicity could be so low that it would not be detectable. It is felt, therefore, that pathogenicity, despite its operational importance, should be and could be excluded from the definition of viruses and even of infection. A virus would be an *entity with an organized infectious phase, containing proteins, possessing one type of nucleic acid, reproduced from its genetic material, unable to grow and to undergo binary fission, and devoid of a Lipmann system. Infectivity of virus is defined as the ability to introduce its genetic material, that is to say a specific information, into a cell.*

These definitions are satisfactory in a way, although they do not mention one of the important characteristics of viruses, namely, their power to modify their host.

When considering a lysogenic bacterium and trying to understand the nature of the relationship between the bacterium and the prophage, one realizes that the prophage is not "neutral." It confers on the lysogenic

bacterium, not only the potential power of producing bacteriophage particle, but also a specific bacterial immunity against homologous bacteriophages. Finally, the prophage may sometimes also be responsible for an alteration of the antigenic constitution of the bacterium. The prophage is a modifying agent.

This ability to modify their host is one aspect of viruses. In the case of the lysogenic bacterium, the modification is not a disease in itself. It might be a disease in the case of malignancy.

In a virus-induced cancer, a normal cell is altered by an oncogenic virus. The malignant cell continues to grow and to divide and, considered by itself is healthy. A cell is, however, not an independent unit, but is a dependent part of an organism.

An organism controls the growth and multiplication of the normal cell but not of the malignant one, which behaves as an independent unit. Its multiplication causes the death of the organism. The oncogenic virus, although it only modifies a cell, kills the organism and is therefore pathogenic.

The action of a virus has to be considered with respect to the multicellular organism as well as to the individual cell. What is a mere harmless alteration at one level might be a fatal disease at another. A virus acting at the level of the dependent and interdependent parts necessarily modifies the independent whole.

## IX. CONCLUSIONS

The student of viruses should remember a few essential principles.

1. Viruses differ from organisms by some essential features: presence of only one nucleic acid, multiplication as genetic material, reproduction from genetic material, inability to grow and to undergo binary fission, absence of a Lipmann system.

2. Viruses differ from cellular organelles by the existence of an organized infective phase.

3. Infectivity of a virus is the ability to introduce its genetic material into a cell. A viral infection is the introduction into a cell or an organism of an entity able to multiply and to reproduce organized infective particles.

4. Viruses may exist in three states: proviral, vegetative, and infective. Reproduction, infectivity, and pathogenicity are never present together during any one of the three phases.

5. One of the characteristics of viruses is their power to impose a permanent alteration of their host cell.

6. The action of a virus has to be considered at the level of the dependent parts of an organism, as well as at the level of the independent organism as a whole.

7. The virus in general is a concept, that is to say, an idea of a class of objects. Because viruses differ from all other categories of entities, we have the right to admit that the category "viruses" has an individuality and perhaps a reality.

8. Viruses are neither organisms nor cellular organelles. Viruses are viruses.

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## Chapter VI

### The Initiation of Bacteriophage Infection

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## I. INTRODUCTION

In the course of their growth cycle, phages undergo a metamorphosis from a metabolically inert extracellular form to an active intracellular form. The pathway of this transition provides the subject matter for the present chapter. The central theme is the remarkable process of *injection*, which serves to introduce the hereditary material of the phage into a bacterial host cell. Sections II and III deal with the composition and organization of the components involved in injection, namely, the extracellular phage particle and the exterior surface of the bacterial cell. In Section IV are described the characteristics of the injection process. Sections V and VI are concerned with physical-chemical and genetic properties of the initial attachment reaction between a phage and a cell (this reaction being of major significance in determining the extraordinary selectivity of the phage-host relationship). Recent chemical and morphological studies bearing on the mechanism of injection are reviewed in the final section.

A review of work on phage infection must depend heavily on information obtained from a single system: phage T2 and its host *Escherichia coli*. Consequently, many of the facts and inferences may not have validity for other phages. It is already apparent that some of the properties of T2 are not found in other unrelated phage strains that have been studied. Nevertheless, we believe that in considering certain important features of phage morphology and behaviour, in particular the chemical and structural relationship of the genetic and nongenetic materials and their respective fates during infection (see Section IV), T2 can serve as a model phage.

The organization of the present chapter has been influenced by the recent appearance of two excellent reviews dealing with many of the topics concerned (Hershey, 1957a; Tolmach, 1957). Therefore we have not found it necessary to cover all points that may be pertinent to the subject at hand, and equal attention has not been given to those covered.

## II. MORPHOLOGY AND COMPOSITION OF BACTERIOPHAGES

### A. Size and Shape

Many properties of phages have been measured in order to determine particle size, notably, rates of diffusion and sedimentation (Putnam, 1953),

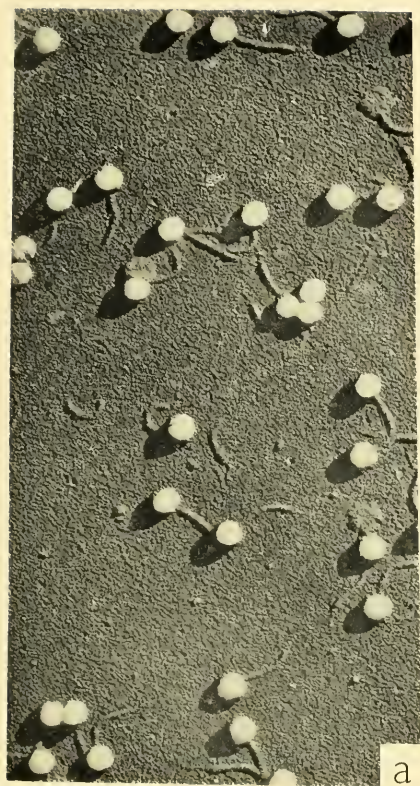


FIG. 1. (a) T5 (Magnification  $\times 48,000$ ) (Courtesy of E. Kellenberger); (b) T2 (Magnification  $\times 31,500$ ) (Courtesy of E. Kellenberger); (c) T3 (Magnification  $\times 63,000$ ) (Courtesy of R. Williams).



filterability (Elford, 1938), and radiation sensitivity (Lea, 1946; Pollard, 1953). However, with the advent of electron microscopy, and its refinement in the hands of T. F. Anderson, R. W. G. Wyckoff, R. C. Williams, and E. Kellenberger, this method now furnishes the definitive values of the dimensions of phages. Fortunately, the sizes of all the known phages fall within the limits of resolution of the most sensitive instruments; the smallest phage, S13, appears to have a diameter of about 300 Å (Tessman, personal communication). Electron micrographs have revealed an array of intricate forms (Fig. 1), each uniformly characteristic of a particular phage or group of related phages. In general, the distinguishing features are a spherical or polyhedral *head* and a narrower cylindrical *tail* (Ruska, 1941; Luria and Anderson, 1942; Anderson, 1953; Williams, 1953). No clear-cut example of a completely tailless phage is known; T3 and T7, once considered tailless, had to be reclassified with the development of more refined techniques (Fraser and Williams, 1953). The tail does not appear to function as a flagellum, since the diffusion constants of several phages can be accounted for satisfactorily by their Brownian motion (Putnam, 1953).

### *B. Chemical and Morphological Components*

#### *1. Purification*

The problem of separating phage particles from the complex mixture of bacterial debris in a lysate may be formidable for some phages (e.g. lambda) and reasonably straightforward for others (e.g. T2). No phage has been crystallized. Most purification procedures rely primarily on enzymatic digestion of bacterial nucleic acids, filtration, precipitation, and differential centrifugation (Herriott and Barlow, 1952). Several sensitive tests for the purity of a preparation are available (in addition to those standard in biochemistry, such as ultracentrifugation and electrophoresis). One is the homogeneity of the particles appearing in the electron microscope. Others are the degree to which the material in the preparation attaches specifically to bacterial host cells, or is precipitable by antiphage and not by antibacterial serum.

The fraction of infective particles in purified phage preparations has been determined for the T-even phages by correlating the number of particles appearing in electron micrographs with the number of plaque-forming units (Luria *et al.*, 1951). At least half of the particles were infective. Similar results have been obtained on unpurified lysates of T2, T4, T5, and lambda (Kellenberger and Arber, 1957). The availability of preparations containing a large fraction of infective particles offers assurance that the chemical and morphological details to be discussed below are pertinent to the mechanisms of phage infection and multiplication.



## 2. Chemical Composition

The most extensive chemical analyses on a phage have been carried out with T2. The total weight per infective particle is  $5 \times 10^{-16}$  grams, consisting mostly of protein and deoxyribonucleic acid (DNA) in about equal amounts. There is no ( $< 0.25\%$ ) ribonucleic acid and no ( $< 0.5\%$ ) lipid (Cohen, 1947; Herriott and Barlow, 1952; Volkin and Astrachan, 1956). The presence of DNA is common to all phages studied (coliphages T1, T2, T3, T4, T5, T6, T7, lambda, and *Salmonella* phage P22), generally in amounts proportional to the relative volumes of the particles (Stent, 1958). (There exist differences in the composition of the DNA of different phages, which are discussed elsewhere in this volume.) Probably the absence of ribonucleic acid also is a common characteristic of phages; T7, like T2, contains no RNA (Lunan and Sinsheimer 1956), and it is not unlikely that the small quantities of RNA reported in preparations of other phages (and in other preparations of T2 and T7) were residual bacterial materials (Putnam, 1953). About 95 % of the phosphorus of T2 (Herriott and Barlow, 1952), but only 71 % of the phosphorus of T7 (Lunan and Sinsheimer, 1956), is located in DNA. The nature of the non-DNA phosphorus fraction is not known.

A few per cent of the material of T2 is soluble in cold trichloroacetic acid (TCA). Three components have been identified in this acid-soluble fraction: a peptide containing only aspartic acid, glutamic acid, and lysine (in contrast to the protein of intact phage, which contains at least 16 amino acids), and two free amino acid-like substances (ninhydrin-positive and probably basic) that are chromatographically different from all amino acids tested (Hershey, 1955, 1957b).<sup>1</sup> These components represent about 1 % of the phage carbon.

## 3. Organization of Protein and DNA

An important clue to the structure of phages was provided by the discovery that the T-even phages lost infectivity when rapidly diluted from concentrated salt solution into water (Anderson, 1949; Anderson *et al.*, 1952), and that, concomitantly, DNA dissociated from the protein (Herriott, 1951). This effect is called osmotic shock, since gradual dilution is ineffective. Electron micrographs of shocked preparations contain particles that retain the overall shape of infective particles but in which the head appears to be collapsed and empty, hence the name *ghosts* (Fig. 2). There also are networks of DNA fibers lying outside of the ghosts (Williams, 1953). In contrast to the DNA in infective particles, the DNA of shocked preparations is sensitive to degradation by DNAase. Ghosts purified by centrifugation after DNAase treatment contain most of the phage protein and are devoid of DNA (Hershey,

<sup>1</sup> These two substances have recently been identified as spermidine and putrescine (Ames *et al.*, 1958).



FIG. 2. T2 after exposure to osmotic shock. Many of the heads have lost the tail structure in addition to DNA. Note that most of the heads are ruptured (Magnification about 42,500). (Courtesy of R. Williams.)

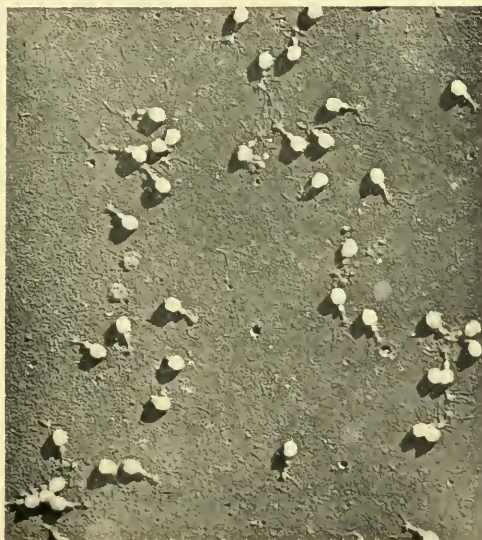


FIG. 3. T2 treated with a solution of ethanol-H<sub>2</sub>O<sub>2</sub> for 10 minutes. Note the morphological details visible in the tail (Magnification about 24,000). (From Kellenberger and Arber, 1955.)

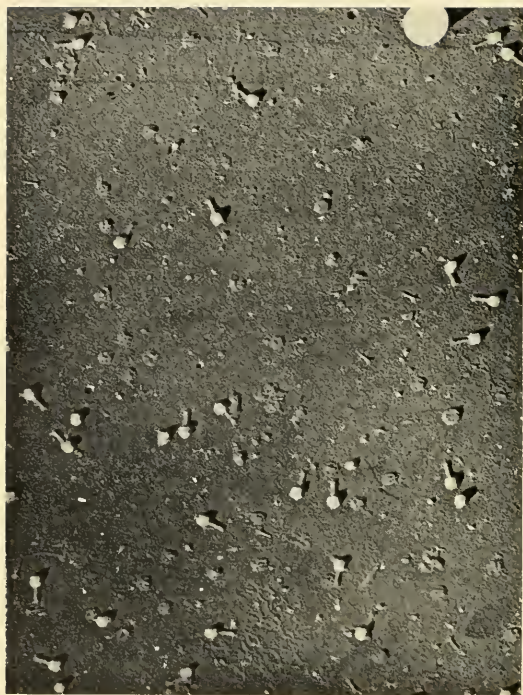


FIG. 4. Crude T2 lysate showing complete phage particles, empty heads, tail cores and fibers, and filaments which may be DNA (Magnification  $\times 19,000$ ). (From Kellenberger and Séchaud, 1957.)



1955). These properties indicate that the heads of intact particles are semi-permeable protein coats that encase the DNA and protect it from enzymatic degradation. The release of DNA by osmotic shock appears to occur through a rupture in the head protein (Fig. 2).

The materials released from the coat of shocked T2 include, in addition to DNA, a protein amounting to about 2 % of the total phage carbon (with about the same amino acid composition as the protein of the intact particle but perhaps richer in lysine), and the three TCA-soluble components described in the preceding section (Hershey, 1955, 1957b).

The viability of phages outside the T-even group is not affected by osmotic shock. This insensitivity to osmotic shock probably is not due to the absence of a protein coat, since DNA-free ghosts of T5 can be formed by heating in a medium deficient in divalent cations (Lark and Adams, 1953), and ghosts of T1, T5, P1, lambda, and a staphylococcal phage have been seen in electron micrographs (Anderson, 1953; Hotchin, 1954; Kellenberger and Kellenberger, 1957). The coats of insensitive phages may be either impermeable or freely permeable to salts, or perhaps capable of withstanding high osmotic pressures.

#### 4. *Structural Units*

Much of our present knowledge about the structure of phage coats has come from high resolution electron micrographs of T2 particles disrupted by freezing and thawing (Williams and Fraser, 1956) or by treatment with a solution of  $\text{H}_2\text{O}_2$ -ethanol (Kellenberger and Arber, 1955). The following components have been detected. (Fig. 3): intact heads (tailless but retaining DNA), ghost heads (devoid of DNA), intact tails, shortened tails with a protruding core 100 Å thick, and tail fibers 60 Å thick which either are free or attached to the tip of a tail or a core. Treatment with DNAase does not alter these forms.

Some of these components are also visible in electron micrographs of crude lysates of T2-infected cells (Fig. 4). It is possible to identify intact phage, heads containing DNA, ghost heads, and in addition, a component called a rod, whose length corresponds to that of the tail (1000 Å) and thickness to that of the core found protruding from the tail of partially disrupted particles (Fig. 3). The rods frequently have fibers attached. It is likely that the rods represent cores that have been released from (or not yet inserted into) tails. Similar rods were first identified in preparations of a staphylococcal phage (Hotchin, 1954).

A current conception of the organization of components in a T2 particle is diagrammed in Fig. 5. It is already apparent that there is considerable anatomical sophistication to this virus.

### *C. Properties of the Components of Phage T2*

#### *1. Attachment to Bacteria*

Several of the components of T2 have been tested for their capacity to attach to sensitive bacteria (Table I). Attachment was determined by centrifuging mixtures of the component and host bacteria, at speeds sufficient to

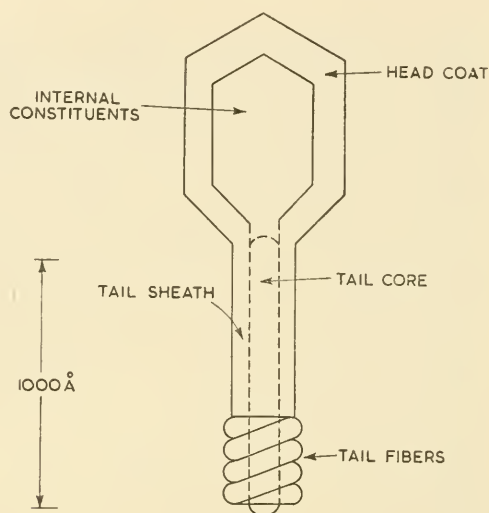


FIG. 5. Model of T2.

TABLE I  
CAPACITIES OF COMPONENTS OF T2 TO ATTACH TO SENSITIVE BACTERIA

| Component                     | Attachment to sensitive cells |
|-------------------------------|-------------------------------|
| Intact phage                  | +                             |
| Ghosts <sup>a</sup>           | +                             |
| DNA <sup>a</sup>              | 0                             |
| Heads <sup>b</sup>            | 0                             |
| Complete tails <sup>b</sup>   | +                             |
| Free tail fibers <sup>b</sup> | +                             |
| Free tail rods <sup>c</sup>   | (+ 0)                         |

+ indicates attachment and 0 lack of attachment.

<sup>a</sup> Hershey and Chase, 1952.

<sup>b</sup> Williams and Fraser, 1956.

<sup>c</sup> Kellenberger and Séchaud, 1957. Appears to consist of two fractions, only one of which attaches. The attaching fraction contains rods that have retained fibers (see Figs. 2, 7); the rods of the nonattaching fraction may have lost their fibers.





a

b

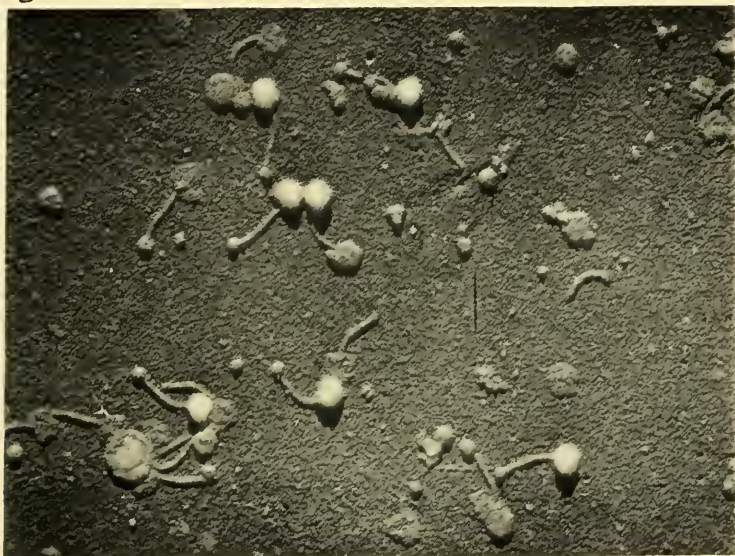


FIG. 6. (a) T5 attached tail-first to a host cell. The heads of several of the particles appear empty, indicating that the DNA has been injected (Magnification  $\times 27,000$ ) (Courtesy of T. Anderson); (b) Mixture of T5 and a cellular component containing the attachment site for T5. For comparison, see Fig. 1(a). The cellular component is the smaller spherical structure that has become attached at the tip of the phage tail. Note that in several particles that have reacted with the cellular component the heads appear empty (Magnification  $\times 41,000$ ). (From Weidel and Kellenberger, 1955.)

FIG. 7. Tail cores of T2 attached by their fibers to a cell wall (Magnification  $\times 33,000$ ). (From Kellenberger and Séchaud, 1957.)

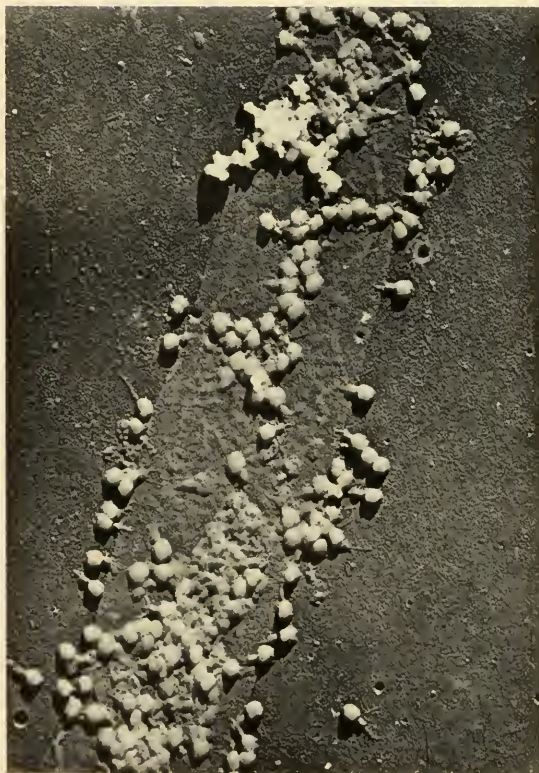


FIG. 8. T2 particles after interaction with a cell wall. Note the alterations in the structure of the phage tail (Magnification  $\times 26,000$ ). (From Kellenberger and Arber, 1955.)

sediment only the cells, and measuring the amount of the component that remained unattached in the supernatant fluid. The amounts of ghost and DNA components were determined by means of specific labeling with  $S^{35}$  and  $P^{32}$ , respectively, and the other components were detected in electron micrographs. The results in Table I indicate that the site for attachment is located on the fibers at the distal end of the phage tail. This conclusion is in line with the earlier finding that phages T2, T1, and T5 attached to cells by the tip of the tail (Fig. 6—Anderson, 1953; Weidel and Kellenberger, 1955).

## 2. Killing of Bacteria

Infection by a virulent phage such as T2 (see Chapter V, page 187) is invariably lethal to a cell. The ability of T2 (and also T5) to kill cells (as measured by the loss of colony-forming activity) does not depend on the production of progeny phage; killing will also occur when phage multiplication has been blocked by exposing the phage before infection to ultraviolet light or to X-rays (Luria and Delbrück, 1942, Watson, 1950). Ghosts of T2, which are devoid of DNA, also kill (Herriott, 1951). Attachment of a single ghost particle is sufficient for this purpose and usually leads within a few minutes to lysis of the cell (Herriott, 1951; French and Siminovitch, 1955; Herriott and Barlow, 1957). Lysis also occurs rapidly when a large number of intact phage attach to a single cell (*lysis-from-without*—Delbrück, 1940b). These three methods of killing and lysing cells (with X-ray-inactivated phage or with ghosts or by the process of *lysis-from-without*) all appear to involve as a first step the formation of cellular structures resembling *protoplasts* (see Section III), which then lyse spontaneously (Watson, 1950; Visconti, 1953; Zinder and Arndt, 1956). Protoplasts do not form in the course of productive infection by a viable T2 particle (otherwise phage production could not occur in the media usually used), which is an indication that an infected cell can block the reaction between a phage coat and the cell surface that yield a protoplast.

The free tail rods found in crude lysates of T2 (Fig. 4) attach to cells but do not kill (Kellenberger and Séchaud, 1957—see Fig. 7). This fact suggests that the phage component responsible for cell killing and lysis (under conditions where there is no phage multiplication, as described in the preceding paragraph) is located in the tail sheath. It may be the enzyme present in the phage tail which degrades cell wall material (see Section VII, B).

## 3. Serology

Two different antigenic components have been identified in T2, one located in the tail and the other in the head of the coat (DeMars *et al.*, 1953; Lanni and Lanni, 1953). The tail contains the antigen specifying the production of



phage-neutralizing antibody, as indicated both by the lack of neutralizing antibody in antiserum made from heads and by the inability of purified preparations of heads to react with neutralizing antibody. Furthermore, T2 particles that have lost their tail fibers and the distal portion of their tail core after treatment with zinc cyanide complexes also lose the capacity to react with neutralizing antibody (Kozloff *et al.*, 1957), which suggests that the tail antigen is located in the distal portion of the tail. Particles neutralized by anti-T2 serum retain the capacity to attach to cells but are unable to kill or to inject their DNA (Nagano *et al.*, 1952; Nagano and Oda, 1955; Tolmach, 1957).

The presence of a head antigen has been detected by complement fixation tests with purified head material (Lanni and Lanni, 1953). It is not known if this antigen is restricted to the head or is also present in the tail. A new complement-fixing activity (without neutralizing activity) has recently been discovered in antiserum made from the material released from T2 by osmotic shock (Levine *et al.*, 1957). The component responsible for this serological activity is a basic protein; its relationship to other phage components has not yet been determined.

### III. THE BACTERIAL SURFACE

Recent studies of the exterior surface of bacterial cells have provided important information about its organization and chemical composition and about the pathways for its synthesis (Work, 1957). The surface structure, or *wall*, has been isolated as a separate component from several bacterial strains after mechanical rupture or chemical treatment of the cell (Salton and Horne, 1951; Weidel *et al.* 1954). Purified walls appear in electron micrographs as empty casings, 100–200 Å in thickness, with contours characteristic of the intact cell (Salton and Williams, 1954). This structure, therefore, must have considerable rigidity. Walls prepared by the above procedures retain the capacity of the intact cell to bind certain phages (Weidel *et al.*, 1954; Salton, 1956). These preparations are chemically complex, containing lipid, sugars, and many amino acids (Work, 1957). Alanine and glutamic acid are usually present in the D as well as the L form, and in certain strains a new amino acid, diaminopimelic acid, replaces some of the lysine.

The surfaces of some bacterial strains are encapsulated by highly polymerized polysaccharide material. It has been found that a phage capable of infecting these cells either contains, or elicits the synthesis of, a hydrolytic enzyme for the capsular material (Adams and Park, 1956).

The cell surface also contains an inner layer, or *membrane*, as revealed in two different ways: by treating intact cells either with lysozyme, which degrades the wall (Weibull, 1953a; Zinder and Arndt, 1956), or with penicillin, which appears to block the synthesis of new wall material (Lederberg, 1956, 1957; Park and Strominger, 1957). Both treatments, when carried out

in a suitable medium, lead to the formation of a dense spherical body, called a *protoplast*, which retains most of the synthetic activities of the intact cell. A protoplast lyses when suspended in water, leaving an empty spherical casing that appears to represent its limiting membrane. The protoplast membrane, unlike the cell wall, does not bind phage (Weibull, 1953a; Brenner and Stent, 1955; Zinder and Arndt, 1956); the fact that some protoplast preparations do bind phage probably can be accounted for by residual wall material. Analysis of the membrane from *Bacillus megatherium* shows that it is composed largely of lipid, protein, and pigments, and contains several enzymes, including the entire cellular cytochrome system (Weibull, 1953b; Storek and Wachsman, 1957). Thus the cell membrane differs from cell wall in morphology, chemical composition, sensitivity to rupture under conditions of high osmotic pressure, and ability to bind phage.

There has been detected in cell walls a subunit composed of a pentapeptide containing alanine, glutamic acid, and lysine (or diaminopimelic acid) linked to the amino sugar, N-acetylmuramic acid. Cells of *Staph. aureus* treated with penicillin excrete this compound in the form of a uridine diphosphate complex, which suggests that protoplast formation with penicillin results from the inability to incorporate the subunit into new cell wall material (Park and Strominger, 1957). The subunit can be removed from the cell wall of *E. coli* by an enzyme present in the tail of T2 (Weidel and Pringosigh, 1957). This reaction may provide the means for penetrating the cell wall barrier in the course of infection (see Section VII). The membrane also presents a barrier to the infecting phage, but there is no information about the reactions employed in penetrating this layer.

#### IV. INJECTION

The first clear indication that different functions were performed by different parts of a phage particle came from an experiment by Novick and Szilard (1951). In examining the phage issuing from cells infected with both T2 and T4, it was found that some of the particles had unusual properties. The host range of these particles (see Section VI) corresponded to that of T4, but all of the progeny produced by these particles exhibited the host range characteristics of T2. It could be concluded that the phage component responsible for host range specificity did not also serve as its own genetic determinant.

The chemical identity of the genetic component was revealed by the blender experiment of Hershey and Chase (1952). It had already been shown for T2 that its DNA was contained in a protein coat, and that attachment occurred at the tip of a long tail on the coat. Hershey and Chase had reason to believe that, during infection, only the DNA, and not the protein coat, entered the cell. In order to test this possibility, they infected cells with T2



labeled with radiosulfur (protein specific, mainly in methionine) or radio-phosphorus (DNA specific), and then exposed the cells to a shearing force in a blender. They found that blending detached from the cells 80 % of the sulfur but only 20 % of the phosphorus of the infecting particle. The ability of the cells subsequently to produce phage offspring was not affected by the treatment. This result indicates that after attachment most of the phage DNA enters the cell, while most of the phage protein remains extracellular. Electron micrographs support this conclusion. They show empty ghosts attached to the surface of infected cells (see Fig. 6); after blending, the ghosts are found detached (Levinthal and Fisher, 1953). The coat of T2 has aptly been called a microsyringe (Hershey, 1953) designed for the injection of its genetically potent contents into a bacterial cell.

The 20 % portion of the DNA phosphorus detachable in the blender appears to be contained largely in intact particles that fail to inject and presumably are noninfective (Hershey and Burgi, 1956). The 20 % portion of the protein sulfur (also protein carbon) that is not detachable is mostly ghost protein that remains attached to the cell surface (Hershey, 1955). There is injected along with the DNA a small amount of non-DNA materials, which include the TCA-soluble components and the internal protein (see Section II).

Blendor experiments on T5 using  $P^{32}$ -labeled phage (Luria and Steiner, 1954) have shown that the DNA of this phage also is injected during infection; injection is slow as compared to T2 and requires addition of  $Mg^{++}$  or  $Ca^{++}$  to the medium. No blender experiments have been reported for any other phages. Less direct evidence for an injection step in T1 has been obtained from other kinds of experiments (Christensen and Tolmach, 1955).

Before proceeding to discuss the steps involved in injection, it may be informative to consider the magnitude of the problem of releasing phage DNA from a protein coat. DNA has the form of a fiber 20 Å in diameter. The DNA in T2, amounting to  $2 \times 10^{-16}$  g., has a total contour length of about 100,000 Å and therefore must be considerably folded to fit within the phage head. (The basic substances in the head (see Section II) may serve to reduce the net negative charge of the DNA and thus aid in bringing about the required folding.) The hollow tube in the tail through which the DNA leaves the head is approximately 100 Å in diameter and 1000 Å in length. It is not known how the DNA is arranged to fit inside the head, but it probably requires careful orientation to pass through the narrow tail.

## V. THE ATTACHMENT REACTION

### A. *Reversibility*

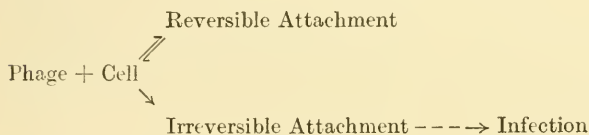
If attachment of a phage particle leads to infection, the over-all reaction will, of course, be irreversible, since the infecting particle cannot be

reconstituted after the DNA has been released from the protein coat. Other irreversible changes preceding the release of DNA also occur (see Section VII). However, when the attachment of T1 and T2 occurs under certain conditions of temperature, ionic environment, or pretreatment of the cells, the attached particles can readily be eluted from the cell surface (Garen and Puck, 1951; Garen, 1954; Christensen and Tolmach, 1955). Since neither the phage nor the cell appears to be changed after this kind of encounter, the reaction is called reversible. Several properties of the reaction indicate that ionic bonds, but not covalent bonds, are involved.

On the basis of results obtained primarily with T1 (Tolmach, 1957) it has been concluded that reversible attachment serves as the first in a sequence of steps on the pathway to infection. Under suitable conditions, reversible attachment would rapidly be followed by irreversible changes culminating in the injection of phage DNA:

Phage + Cell  $\rightleftharpoons$  Reversible Attachment  $\rightarrow$  Irreversible Attachment  $---\rightarrow$  Infection

A different interpretation has also been proposed (Hershey, 1957a), in which reversible attachment is considered, not as one of the steps of infection, but rather as an independent competitive reaction:



The evidence pertaining to the role of reversible attachment in the process of phage infection has been critically discussed in two recent reviews (Tolmach, 1957; Hershey, 1957a).

### *B. Influence of Temperature*

The velocity of phage attachment to host cells is almost as rapid at 0 as at 37°C., but other characteristics of the reaction may vary markedly in this temperature range. With T1 and T7, attachment changes from an irreversible reaction at 37°C. to one that is largely reversible at 0°C., which indicates that the first of the irreversible attachment reactions has a high temperature sensitivity and therefore involves covalent bond formation (Garen and Puck, 1951; Garen, 1954; Mackal and Kozloff, 1954; Christensen and Tolmach, 1955). With T2, in contrast to T1 and T7, attachment is irreversible at 0 as well as at 37°C. However, at the lower temperature, attachment of T2 does not lead to infection. Most of the particles attaching at 0°C. eject their DNA into the medium, which accounts for the irreversibility of the reaction, and the cells remain viable (Puck, 1953; Adams, 1955). Evidently the mechanism for ejecting phage DNA from the protein coat after attachment has occurred

is less temperature-sensitive in T2 than in T1 or T7. (Other differences in this mechanism are discernible in the interaction of T2 and T1 with an ion exchange resin; DNA is released from T2, but not from T1, after the phage has attached to a resin—see Section V, E). It is noteworthy that, except for the reversibility behavior, the attachment reactions of T2, T1, and T7 respond similarly to a change in temperature; when attachment occurs at 0 instead of at 37°C., the host cell is not killed and the phage DNA is not injected into the cell. The temperature-sensitive step which is prerequisite for cell killing and DNA injection has not been identified.

### *C. Influence of the Medium*

Attachment does not occur in distilled water, probably because both the phage and cell surfaces carry a net negative charge. Attachment usually begins upon addition of an inorganic salt (Puck *et al.*, 1951). The salt requirement is phage-specific. For example, T1 attaches at optimal velocity in a  $10^{-3}$  M solution of a divalent cation salt (of  $Mg^{++}$ ,  $Ca^{++}$ ,  $Ba^{++}$ , or  $Mn^{++}$ ), or in a  $10^{-2}$  M solution of a monovalent cation salt (of  $Na^+$ ,  $K^+$ ,  $Li^+$ , or  $NH_4^+$ ), while attachment of T2 is optimal in a  $10^{-1}$  molar solution of a monovalent cation salt, divalent cations being ineffective. Since any one of a variety of cations are effective with T1, apparently their only function is to neutralize repulsive electrostatic forces between a phage particle and a cell. With T2, other cation-specific factors must be important, but these have not been identified.

Attachment generally is measured at pH 7. The effect of changing the pH has been determined for T1 and T2. Both phages attach best in the pH range from 6 to 10, and fail to attach at the pH extremes of 4.8 and 12. This behavior suggests that ionized carboxyl and amino groups are needed for attachment of these phages (Puck and Tolmach, 1954).

### *D. Tryptophan Requirement*

Certain strains of the T-even phages cannot attach in a synthetic medium unless first "activated" by exposure to L-tryptophan (Anderson, 1948). The amino acid requirement for activation is remarkably specific; most other amino acids, including D-tryptophan, are ineffective, and a few work only at a concentration considerably greater than is needed with L-tryptophan. Some strains require tryptophan for attachment at 15 but not at 37°C.; others require calcium ions in addition to the amino acid (Delbrück, 1948). Activation appears to require the binding of five molecules of tryptophan to a phage particle, presumably at a critical region of the tail (Stent and Wollman, 1950). The activating effect of tryptophan is rapidly and completely reversed when tryptophan is removed from the medium. Recently it

was discovered that the tryptophan-activated state can be stabilized against deactivation in the absence of tryptophan by a component of antiphage serum which does not have phage-neutralizing activity (Jerne, 1956).

Another effect of tryptophan on the strains that require activation is to render the tail of the phage more sensitive to irreversible structural changes inducible by heating (Cheng, 1956) or by exposure to zinc cyanide complexes (Kozloff *et al.*, 1957), which implies that tryptophan may exert its activating effect by opening or weakening some of the bonds in the tail proteins. This interpretation gains support from the finding that the protein-denaturing agent, urea, also activates attachment of these strains although, unlike tryptophan, it causes additional changes which are lethal (Sato, 1956). The role of activation in the attachment reaction may be the unwinding of tail fibers to some extent in order to expose them to contact with the cell surface. Accordingly, the requiring and nonrequiring strains may differ by secondary protein linkages that serve to maintain the fibers in a tightly folded condition.

### *E. Attachment to Noncellular Surfaces*

Phages attach, not only to specific host cells, but also to ion exchange surfaces, such as glass and resins. In certain important respects attachment to glass or a cation exchange resin closely resembles attachment to cells (Puck *et al.*, 1951; Puck and Sagik, 1953). For example, both reactions have similar specific requirements for inorganic salts and, in the case of the tryptophan-requiring strains of T4, for L-tryptophan. The tryptophan effect offers compelling evidence that the same sites on the phage are involved in both reactions. Attachment of T1 to glass or resin is a reversible reaction, while attachment of T2 to resin leads to the release of DNA from the protein coat.

There are differences as well as similarities between the behavior of phage toward ion exchangers and cells. For example, the salt requirement for attachment of T2 to a cation exchange resin can be satisfied either by monovalent or divalent cation salts, but only by the monovalent cation salts for attachment to cells. The biological implications one associates with the attachment of phage to ion exchange surfaces depend, of course, on whether greater emphasis is placed on such points of difference between ion exchangers and cells (Hershey, 1957a), or on the similarities (Puck *et al.*, 1951; Puck and Sagik, 1953; Puck, 1953; Tolmach, 1957).

Phage also attach to an anion exchange resin. This reaction, in contrast to the reaction with a cation exchanger or a cell, occurs as well in distilled water as in a salt medium, showing that salts are required only for attachment to a negatively charged surface.



### F. Rate of Attachment

It was first proposed by Schlesinger (1932) that the phage attachment reaction could be treated theoretically in terms of diffusion equations previously developed to describe coagulation of colloidal particles. Schlesinger defined a velocity constant  $k$  by the equations:

$$P + C \xrightarrow{k} PC$$

$$\text{velocity of attachment} = \frac{d(PC)}{dt} = -\frac{d(P)}{dt} = k(P)(C)$$

where P is unattached phage, C is the cell, and PC is attached phage.

and obtained accurate measurements of  $k$  under various experimental conditions. These values were compared to the maximum theoretical value of  $k$  to be expected if every contact between a phage and a cell, arising from random diffusion of the phage particles, resulted in attachment. The theoretical value was estimated by assuming that a cell provides a stationary surface which acts as a perfect phage absorber; since every phage particle reaching the cell surface attaches, the phage concentration of unattached phage at the cell surface will be zero (Delbrück, 1940a). A concentration gradient of phage particles will then arise in the near vicinity of the cell surface, which will bring about a net flow (by diffusion) of particles towards the cell. The velocity of flow will be determined by the concentration gradient and the diffusion constant of the phage. The maximum value of  $k$ , as calculated theoretically in this manner, is close to the maximum experimental values obtained with several phages; this result has led to the conclusion that essentially every "collision" (i.e., surface contact) between a phage and a cell leads to attachment (Puck *et al.*, 1951; Stent and Wollman, 1952; Tolmach, 1957).

This conclusion must be reconciled with the requirement that a collision between a phage particle and a cell can result in attachment only if the phage tail is brought into juxtaposition with an attachment site on the cell. This requirement should limit the chance that a random collision will be effective, since collisions should not invariably occur at an attachment site or in a tail-first orientation. These steric considerations make it unlikely that the attachment reaction can achieve perfect efficiency, but it is not clear to what extent they limit the maximum attainable efficiency, since there also are compensating factors. For one thing, there are at least several hundred separate sites on an individual cell for binding a particular phage (Watson, 1950; Garen, 1954), and for another, probably a significant fraction of collision orientations enable the tail fibers of the phage to come into contact with the cell surface, especially since the fibers appear to be unwound to a large extent (Fig. 7).



It was pointed out recently (Hershey, 1957a) that it may not be valid to draw any conclusions about the collision efficiency of the phage attachment reaction on the basis of diffusion theory alone. The Schlesinger calculation can be used to evaluate the rate of collisions between phage particles and cells only in cases where attachment occurs with perfect efficiency. If every collision does not lead to attachment, the phage concentration at the cell surface becomes finite instead of zero, and the rate of collisions can no longer be evaluated by the method of Schlesinger. It has been concluded that the collision efficiency of attachment of T1, T2, and T4 is essentially perfect because of the close agreement between the measured rate of attachment and the theoretical maximum rate calculated from the Schlesinger equation. However, a possible discrepancy between the measured and theoretical rates of as much as 50 % has not been excluded. A discrepancy of this magnitude does not mean that the collision efficiency also is 50 %. The efficiency may be considerably lower, since a phage particle that diffuses to the cell surface, but does not attach on the first collision, is likely to collide several times more before diffusing away.

## VI. GENETIC CONTROL OF ATTACHMENT SPECIFICITY

### *A. Resistant Cell Mutants*

In a large population of bacterial cells, all descended from a single phage-sensitive parent, there can usually be found some cells that are phage-resistant (i.e., are not killed by exposure to the phage). Resistant cell strains have been shown to originate from random mutational events that occur during the multiplication of the sensitive strain (Luria and Delbrück, 1943). In general, it has been possible to obtain resistant cell mutants with all the known phage-host systems. When the parental strain is sensitive to many different phages, as with *E. coli* B, sensitive to the seven T-phages, a mutation may impart resistance only to one phage (e.g. B/6, resistant only to T6) or simultaneously to several (e.g., B/3, 4, 7, resistant to T3, T4, and T7). Resistance to several different phages can also develop by successive independent mutations.

A phage generally cannot attach to the bacterial mutant that is resistant to it. This kind of cell mutation, therefore, must affect the cell surface. The fact that resistance may be selective for only one of the phage strains active on a cell indicates that different strains may attach to different cell sites and that the surface modifications responsible for resistance are localized at or near the attachment sites for a particular phage. In cases where resistance can develop simultaneously to several different phage strains, the phages involved in the pattern of resistance probably use overlapping attachment sites

(Puck, 1953). A mutation to resistance to one phage strain frequently decreases the rate of attachment of another phage, although not sufficiently to impart resistance to the other phage as well (Luria, 1945a,b). Thus, the effect of a mutation may be more extensive than is indicated by the sole criterion of resistance. The various patterns of resistance that can arise in a sensitive cell make it apparent that the structure of the cell surface is subject to many different modifications through mutations.

Resistance to T1 in *E. coli* B can rise in two ways (Garen and Puck, 1951). In one kind of mutant, B/1, irreversible attachment is blocked but reversible attachment remains unimpaired. In another mutant, B/1, 5, neither attachment reaction can occur. All other resistant mutants that have been studied are, like B/1, 5, incapable of interacting either reversibly or irreversibly with the phage. It should be recalled that since these mutants are selected for total resistance to a phage, a selection is at the same time being made for mutations that drastically affect the attachment reaction. There is likely to exist another important class of mutants having more subtle surface modifications. The rate of phage attachment to such mutants might be slow but still sufficient to prevent colony formation in the presence of the phage. These mutants would therefore remain undetected by the usual procedures.

Attachment reactions may also be affected when a cell is lysogenized (Burnet and Lush, 1936; Bradley and Boyd, 1952; Boyd, 1954). This process differs from the random mutations discussed above, in that the mutagenic agent is known to be the lysogenizing phage, and each lysogenized cell is modified in the same ways. We shall not be concerned here with the general aspect of phage resistance in lysogenic cells, since this usually involves intracellular reactions subsequent to attachment and injection (see Chapter 5). The specific effects of lysogeny on attachment may be twofold: the lysogenic cell may lose the capacity to react with the phage strain used to lysogenize, and it may also gain the capacity to react with a different phage. For example, *Salmonella* phage E15 can attach only to cells of *S. anatum* that are non-lysogenic for E15, while another phage, E34, can attach only to the cells that are lysogenic for E15 (Uetake *et al.*, 1958). In another system, that of *S. typhimurium* and phage P22, lysogenization decreases the rate of (but does not completely block) attachment of P22 (Garen and Zinder, personal communication). However, lysogeny does not always influence attachment of the carried phage strain; coliphage lambda can attach equally well to lysogenic (for lambda) and nonlysogenic *E. coli* K12 (Lieb, personal communication).

It was pointed out many years ago by Burnet (1930) that the surface antigens of the cell frequently determine attachment specificity. A particularly clear example is provided by the serological changes in *S. anatum* which result from lysogenization and are associated with the development of resistance to phage. Cells lysogenic for phage E15 lack a surface antigen carried by

the nonlysogenic strain, and also contain a new surface antigen not found in the nonlysogenic strain (Uetake *et al.*, 1958). The correspondence of these antigenic changes with the changes in attachment specificity (cited in the preceding paragraph) suggests that the attachment sites for the phages concerned are located on the antigens.

The most extensive analysis of the serological and chemical effects of mutation to resistance has been carried on mutants of *Shigella sonnei* and *E. coli* resistant to phages T3, T4, and T7 (Goebel and Jesaitis, 1952; Weidel *et al.*, 1954). The sensitive and resistant strains of *Sh. sonnei* differ serologically. A purified antigen isolated from the sensitive strain reacts with the three phages, but the antigen from the resistant strain is inactive. These antigens are composed of a complex of lipocarbohydrate and protein. When the lipocarbohydrate is isolated as protein-free hapten from the sensitive strain, it retains the capacity to react with the three phages, but the corresponding haptenic component from the resistant mutant does not. It is of particular interest that chemical differences have been detected between the carbohydrate moieties of these lipocarbohydrate components from sensitive and resistant strains of *Sh. sonnei* and *E. coli*. Further detailed studies of this kind should contribute significantly to an understanding of the chemical basis of attachment specificity.

### *B. Host-Range Mutants of Phages*

By use of phage-resistant cell mutants, it usually is possible to isolate phage mutants that can attach to and infect the resistant cells. This type of mutant, called host range or *h*, can be selected for in a population of parental *h*<sup>+</sup> particles by plating for plaques on the resistant cells (Luria, 1945a,b). The converse mutation, from *h* to *h*<sup>+</sup>, also occurs. The *h*<sup>+</sup> particles can be detected, although not selectively, by plating on a mixture of resistant and sensitive cells; plaques of *h*<sup>+</sup> particles are turbid, in contrast to the plaques of *h* particles, which are clear (Hershey and Davidson, 1951; Streisinger and Franklin, 1956).

The genetic characteristics of host-range mutations in T2 have recently been analyzed in fine detail (Streisinger and Franklin, 1956). Genetic crosses (see Chapter VIII, page 281) were run on two classes of mutants. One class consisted of *h*<sup>+</sup> mutants originating from independent mutational events in an *h* strain. The other class consisted of *h* mutants originating from independent mutational events in several *h*<sup>+</sup> strains. In pairwise crosses between *h*<sup>+</sup> mutants from the first class, *h* recombinants were usually formed. This result shows that an *h*<sup>+</sup> mutation can arise at many separate locations on the chromosome of an *h* strain. (All the *h*<sup>+</sup> mutants studied have been found to be closely linked, that is, the mutations cluster within a limited region of the phage chromosome.) The behavior of the second class of mutants, however,

was quite different. In crosses between  $h$  mutants, no  $h^+$  recombinants could be detected. Since all of the  $h$  mutants appear to be genetically identical by this sensitive test, a mutation to  $h$  in any  $h^+$  strain must restore the original  $h$  genotype. In summary: the capacity of an  $h$  strain of T2 to attach to B/2 cells can be lost by any one of several different  $h^+$  mutations occurring within a limited region of the chromosome, and this capacity can be regained only by a reverse mutation which restores the original  $h$  genotype.

Accordingly, the host-range region of the chromosome of an  $h$  strain probably determines a specific configuration at the tip of the phage tail which is required for attachment to B/2. The modifications of this tail configuration introduced by  $h^+$  mutations cause the loss of all affinity for B/2. It should be noted that the  $h^+$  mutants are selected for their ability to infect B cells as well as for their inability to infect B/2 cells. This means that the  $h^+$  mutations must modify the phage tail in a way that specifically blocks attachment to B/2 but not to B. There are likely to occur other mutations which also block attachment to B and therefore go undetected. With some of the  $h^+$  mutants the rate of attachment to B is in fact decreased, but not sufficiently to prevent plaque formation (Streisinger and Franklin, 1956).

The  $h^+$  mutants of T2 may differ from the  $h$  parent in their susceptibility to inactivation by heating (Hershey and Davidson, 1951; Streisinger and Franklin, 1956); the heat sensitivity may be greater or lesser than it is for the parental  $h$  strain. Heat-inactivated particles cannot attach to sensitive cells, probably because the structure of the tail tip has been altered through protein denaturation. The heat inactivation properties show that different  $h^+$  mutations modify the tail structure in different ways, although all of the mutations bring about the loss of affinity for B/2.

The specific effect of  $h^+$  mutations on attachment to resistant cells can be accounted for formally in either of two ways. One way is to assume that there are two separate kinds of attachment sites on the phage, one for attaching to sensitive cells and the other to resistant cells (perhaps located on different tail fibers), and that each site is under independent genetic control. The  $h^+$  mutations would then modify only sites of the second kind. The other way is to assume that there is only one kind of site on the phage for attaching both to sensitive and to resistant cells, and that certain modifications of this site caused by  $h^+$  mutations selectively destroy the capacity to attach to resistant cells. (There also are two corresponding ways to account for the difference between phage-sensitive cells and phage-resistant cell mutants; by postulating either two kinds of cellular sites under independent genetic control, one for binding  $h$  particles and the other for binding  $h^+$ , or a single kind of site susceptible to mutational modifications that selectively destroy its affinity to bind  $h^+$  particles.)



The procedure described above, of first isolating a phage-resistant cell mutant and subsequently isolating a host-range mutant of the phage that can infect the resistant cell, has been extended with T2 and *E. coli* B, as shown in Table II (Luria 1945a,b; Baylor *et al.*, 1957). The resistant cells

TABLE II

HOST-RANGE MUTANTS OF T2 AND CORRESPONDING T2-RESISTANT MUTANTS OF  
*E. coli* B  
(From Baylor *et al.*, 1957)

| Phages                   | Hosts |     |               |                           |
|--------------------------|-------|-----|---------------|---------------------------|
|                          | B     | B/2 | B/2/ <i>h</i> | B/2/ <i>h</i> , <i>h'</i> |
| T2 <i>h</i> <sup>+</sup> | +     | 0   | 0             | 0                         |
| T2 <i>h</i>              | +     | +   | 0             | 0                         |
| T2 <i>h'</i>             | +     | +   | +             | 0                         |
| T2 <i>h''</i>            | +     | +   | +             | +                         |

Key to symbols:

+ Indicates plaque formation.

0 Indicates failure of plaques to appear.

B/2, B/2/*h*, and B/2/*h*, *h'* were obtained by plating, respectively, B with an excess of T2*h*<sup>+</sup> and B/2 with an excess of T2*h*, and selecting surviving colonies. The phage mutants *h'* and *h''* were obtained from plaques formed on plating, respectively, *h*<sup>+</sup> with B/2/*h* and *h'* with B/2/*h*, *h'*. It is evident from these results that both a phage and a cell possess the genetic capacity for modifying their surface structure in many ways.

Host-range mutants of phage have not been found with all systems. For example, attempts to isolate a T1*h* mutant that can infect B/1, 5 have failed, although *h* mutants that infect B/1 are readily found (Luria, 1945a,b). Similarly, T4*h* mutants are known only for K12/4 but not for B/4 (Benzer, personal communication).

Another type of host-range mutant, called *ht*, has been detected in *h*<sup>+</sup> strains of T2 (Hershey and Davidson, 1951; Baylor *et al.*, 1957). Mutants of this type form turbid plaques on B/2 (plaques from *h* are clear). A strain of *ht* attaches to B/2 at a slower rate than *h* (probably accounting for the plaque turbidity), but both strains attach at about the same rate to sensitive B cells (Hershey and Davidson, 1951). An *ht* mutation, therefore, partially restores the *h* phenotype to an *h*<sup>+</sup> strain. Independent *ht* mutations in an *h*<sup>+</sup> strain occur at many separate positions scattered over almost the entire phage chromosome, and in this way differ from *h* mutations, which can occur



only at one specific position in the  $h^+$  strain. A striking feature of  $ht$  mutations is their cumulative effect on the rate of attachment to B/2. For example, starting with an  $h^+$  strain which is incapable of attaching to B/2, a single  $ht$  mutation enables attachment to occur at a slow rate, and an accumulation of two or three different  $ht$  mutations increases the rate to the maximum value obtained with an  $h$  strain. Thus, the attachment properties achieved in a single mutational step from  $h^+$  to  $h$  can be approached in several additive steps via  $ht$  mutations. In addition to the effects of  $ht$  mutations on  $h^+$  strains, the presence of these mutations in an  $h$  strain extends the host range. For example, B/2/ $h$  is resistant to T2 $h$  (see Table II) but is sensitive to an  $h$  strain which also harbors  $ht$  mutations (Baylor *et al.*, 1957). Host-range mutants which appear to be similar to the  $ht$  mutants of T2 have been found with T3 (Fraser and Dulbecco, 1953).

It becomes evident from the preceding discussion that the attachment specificity of a phage is under the control of a highly complex pattern of genetic factors. This system offers the intriguing problem of elucidating the chemical and morphological bases of the phenotypic variations resulting from host-range mutations, many of which must be exceedingly subtle.

#### *C. Genetic Control of Tryptophan Requirement*

The unique requirement of certain strains of the T-even phages for L-tryptophan as a cofactor for attachment (see Section V, D) has been subjected to a genetic analysis (Brenner, 1957). In pairwise crosses between independently isolated tryptophan-requiring strains of T4 (called  $c$ ), tryptophan-independent recombinants ( $c^+$ ) were produced, showing that mutations to  $c$  can arise at different positions on the chromosome of a  $c^+$  strain. The  $c$  mutations studied were found to be closely linked, and in this respect are similar to the host-range  $h^+$  mutations which also cluster within a single region of the chromosome (separated from the region of the  $c$  mutations). The effect of  $c$  mutations probably is on the arrangement of the tail fibers (see Section V, D); this effect may differ among different mutants, as indicated by the requirement for calcium ions, in addition to tryptophan, with some of the mutants, and by the temperature-dependence of the tryptophan requirement with other mutants (Delbrück, 1948; Brenner, 1957). Thus, the genetic and phenotypic properties required by the T-even phages to achieve tryptophan independence appear to be quite specific, since the transformation from independence to dependence can occur in various ways as a result of different  $c$  mutations.

#### *D. Phenotypic Mixing*

The host-range properties of some of the progeny formed in crosses between a T2 and a T4 parent are unusual for two reasons. First, as was

discussed in Section IV, there are particles possessing the host-range phenotype of one of the parents and the host-range genotype of the other parent, indicating that phage tails (determining host-range phenotype) and DNA (determining genotype) are paired unspecifically from components derived from both parents during formation of phage progeny. Similar results have also been obtained for the tryptophan-dependence characteristic in crosses between dependent and independent strains (Brenner, 1957). Second, there are particles possessing the host-range phenotype (but not the genotype) of *both* parents (Streisinger, 1956), as recognized by their capacity to attach both to B/2 and B/4 cells. The existence of these phenotypically hybrid particles shows that at least two kinds of attachment sites can be present in a single particle (perhaps located on separate tail fibers) and that each site can function independently.

The relationship of tryptophan dependence to host range has been studied by crossing strains of T2 and T4 having different tryptophan requirements (Brenner, 1957). Phenotypic analysis of the immediate progeny from the crosses showed that the host-range and tryptophan-dependence characteristics were paired almost at random in an individual particle; that is, a particle with the host-range phenotype of one of the parents had almost a random chance of having the tryptophan-requirement phenotype of the other parent. However, these two characteristics were found to be genetically linked; that is, a particle with the host-range genotype of one of the parents was likely to have also the tryptophan-dependence genotype of that parent. These results indicate that tryptophan-dependence and host-range phenotypes are determined by separate components of the phage coat (corresponding to the separate regions of the chromosome that determine the two genotypes, as discussed above).

## VII. ON THE MECHANISM OF INVASION

### *A. Alterations of Phage Tail Structure during Invasion*

#### *1. Morphological Changes*

In 1955, Kellenberger and Arber published electron micrographs (Fig. 8) showing the changes in intact T2 (and T4) resulting from interaction with cell walls prepared by the procedure of Weidel (1951). It can be seen that a radical alteration of the tail structure has occurred. The fibers at the distal portion of the tail are no longer visible, and there is revealed the core of the tail. The proximal portion of the tail is now about 40 % thicker than in intact particles. Essentially the same alterations in the phage tail after interaction with cell walls have since been observed by Brown and Kozloff (1957). In Fig. 8 it should be noted that, although the tails are altered, the heads

appear to contain DNA, indicating that injection does not occur with the type of cell wall preparation used in these experiments.

## 2. Removal of Tail Fibers

The tail of T2 is composed of several protein components (see Section II), and each probably plays a specific role during invasion. The total amount of protein in T2 corresponds to a molecular weight of 125,000,000. It has been calculated that, of the total phage protein, the tail structure represents 12 %, with about 3 % in the tail core and 1 % in the tail fibers (Kozloff *et al.*, 1957). Since there are 4 or 5 fibers, each fiber has a molecular weight of perhaps 250,000. It is of interest that, by radiation studies, Pollard and Setlow (1956) calculated that the attachment of T2 to its host cells is mediated by a portion of the T2 structure having a molecular weight of 186,000.

The only well-established covalent bonds for linking separate polypeptide chains, such as the components of the phage tail, are the disulfide and phosphodiester bonds. The evidence on the nature of the bonds in the phage tail implicates a sulfur-containing bond (Kozloff *et al.*, 1957). It appears not to be a disulfide bond. The evidence also argues against a phosphate ester bond, a peptidic bond, or an oxygen ester or ether bond. On the basis of the morphological changes of the phage particle caused by various chemical and physical treatments, it seems likely that thiolester bonds are the main bonds between the tail fibers and the rest of the tail. Every treatment known to disrupt thiolester bonds causes the tail fibers to become partially unwound, or completely unwound, or removed from the phage tail.

It should be emphasized that this type of evidence for a thiolester bond as the main structural link in the phage tail is quite circumstantial. However, it is difficult to obtain direct evidence. From an estimation of the amount of cysteine sulfur in the tail fibers, it was calculated that there are at most 300 thiolester bonds in the tail structure (Kozloff *et al.*, 1957). These would be difficult to detect because the turbidity of phage preparations greatly limits the use of colorimetric methods.

Table III lists the effect of various chemical and physical treatments on the structure of T2; some of the typical morphological alterations are illustrated in Fig. 9. The details of the treatments are given in the references. Papain, which has thiolesterase activity, is the only enzyme so far reported to affect the structure of T2 phage. The fact that several reagents which have little or no effect on disulfide bonds can alter the tail structure would seem to eliminate the possibility that disulfide bonds are the main structural cross links. These reagents are pH 10 buffer, *N*-ethyl-maleimide, and  $\text{NH}_2\text{OH}$ . The removal of the tail fibers in pH 10 buffer would also eliminate hydrogen bonding between SH and amino groups as the main linkage, since this bond is not broken in alkaline solution (Benesch *et al.*, 1954). In general, the degree





a



b



c



d

FIG. 9. T2 after treatment with various chemicals: (a)  $\text{Cd}(\text{NH}_3)_6^{2+}$  removes tail fibers (Magnification about  $\times 21,000$ ) (From Kozloff *et al.*, 1957); (b)  $\text{NH}_2\text{OH}$  at pH 6.6 inactivates T2 and causes unwinding of tail fibers (Magnification about  $\times 34,000$ ); (c) L-arginine disrupts the head and the tip of the tail (Magnification about  $\times 20,000$ ) (From Kozloff *et al.*, 1957); (d) *N*-ethyl maleimide removes tail fibers (Magnification about  $\times 32,000$ ).

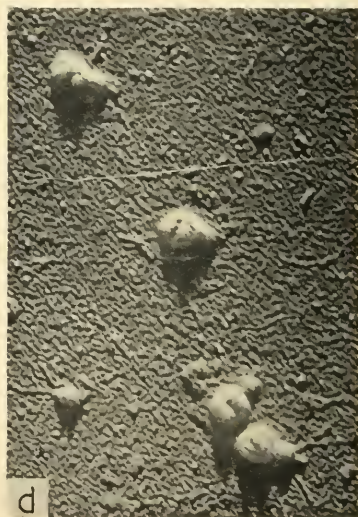
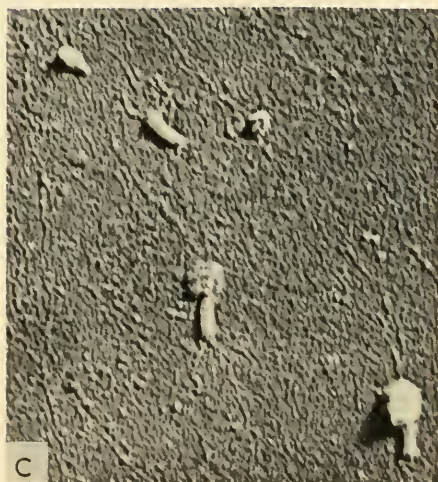
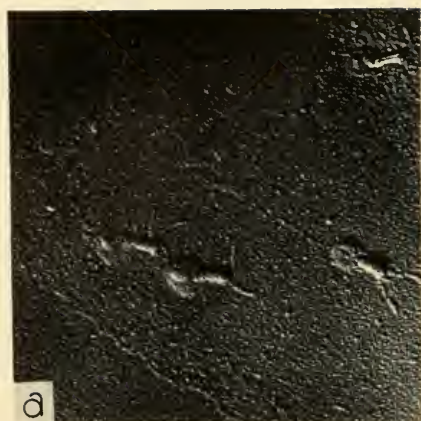


FIG. 10. (a) T2 after incubation for 3 hours with glycine buffer at pH 10 (Magnification about  $\times 40,000$ ); (b) T2 after incubation for 6 hours with glycine buffer at pH 10 (Magnification about  $\times 40,000$ ); (c) T2 treated first for 4 hours with glycine buffer at pH 10 and subsequently with  $\text{KH}_2\text{PO}_4$  to reduce the pH to 7.3. Note the contracted tail in an upper corner of the figure (Magnification about  $\times 53,000$ ); (d) T2 after interaction with cells in the presence of 0.001 *M* EDTA. Note the partially contracted tails (Magnification about  $\times 47,000$ ).



of alteration of the tail appears to be related to the efficiency of the reagent in breaking thiolester bonds.

TABLE III

CHEMICAL AND PHYSICAL TREATMENTS WHICH ALTER THE STRUCTURE OF T2

| Treatment                               | Alteration                     |                                      |              |                      | References                   |
|---|--------------------------------|--------------------------------------|--------------|----------------------|------------------------------|
|   | Tail fibers unwound or removed | Contraction of proximal tail protein | DNA released | Head protein altered |                              |
| Zn(CN) <sub>3</sub> <sup>-</sup>        | +                              | +                                    | 0            | 0                    | Kozloff <i>et al.</i> , 1957 |
| Zn(CN) <sub>3</sub> <sup>-</sup>        |                                |                                      |              |                      |                              |
| + Lysine                                | +                              | +                                    | +            | 0                    | Kozloff and Lute, 1957b      |
| H <sub>2</sub> O <sub>2</sub> , 10 min. | +                              | +                                    | 0            | 0                    | Kellenberger and Arber, 1955 |
| H <sub>2</sub> O <sub>2</sub> , 60 min. | +                              | +                                    | +            | +                    | Kellenberger and Arber, 1955 |
| Thioglycollate                          | +                              | +                                    | +            | +                    | Kozloff <i>et al.</i> , 1957 |
| N-ethyl —                               |                                |                                      |              |                      |                              |
| maleimide                               | +                              | +                                    | 0            | 0                    | Kozloff, 1958                |
| OH <sup>-</sup> (pH 10)                 | +                              | 0                                    | +            | +                    | Kozloff, 1958                |
| Arginine                                | +                              | +                                    | +            | +                    | Kozloff and Lute, 1957b      |
| NH <sub>2</sub> OH                      | +                              | 0                                    | 0            | 0                    | Kozloff, 1958                |
| Papain                                  |                                |                                      |              |                      |                              |
| (thiolesterase)                         | +                              | +                                    | 0            | 0                    | Kozloff <i>et al.</i> , 1957 |
| Gentle heat                             | +                              | +                                    | +            | +                    | Cheng, 1956                  |
| Frozen-thawed                           | +                              | +                                    | +            | +                    | Williams and Fraser, 1956    |
| Osmotic shock                           | 0                              | 0                                    | +            | ?                    | Herriott and Barlow, 1957    |

Key to symbols:

+ Indicates that the treatment is effective.

0 Indicates that no visible change occurred.

? Indicates that although no visible change occurred, there is other evidence that the treatment had some effect.

Although it is known that other treatments, such as urea (Cohen, 1947), *p*-chloro-mercuribenzoate (Puck and Tolmach, 1954), and formaldehyde (Tolmach and Puck, 1952) inactivate T2 and presumably alter its structure, no electron micrographs are available. Therefore these results are not tabulated.

The effects of these reagents also reveal some properties of the phage head protein. The destruction of the head protein by thioglycollate and prolonged peroxide treatment (but not brief peroxide treatment) suggests that one of the factors in maintaining the structure of the head is disulfide bonding. It also appears that the highly organized head structure can be disrupted by

the usual denaturing agents which break hydrogen bonds, such as heat and alkali. The phage DNA can escape when the phage is exposed to arginine, heat, or alkali, even though the head protein remains attached to the tail structure. There is very little evidence on the nature of the bonds attaching the head structure to the tail. The head protein can be almost completely separated from the tail by prolonged alkali treatment, but this property does not clearly implicate any particular bond.

### 3. *Role of Zinc*

The highly specific action of complexes of the zinc group metals on the tail proteins of T2 bacteriophage (Kozloff and Henderson, 1955), as listed in Table III, appeared to duplicate the action of the cell wall on the phage particle. In 1957, Kozloff and his co-workers (Brown and Kozloff, 1957; Kozloff and Lute, 1957a) furnished direct evidence that zinc, tightly bound to the cell wall, was necessary for the alteration of the phage tail during invasion.

It was shown that there was an enzyme in T2 which could partially degrade the host cell wall (Barrington and Kozloff, 1954, 1956). However, intact T2 was enzymatically inactive and the enzymatic activity was exposed only after the tail fibers were removed (Brown and Kozloff, 1957). Zinc tightly bound to the cell wall was necessary for the exposure of this enzyme during phage-cell wall interaction (Kozloff and Lute, 1957a). Zinc is known to be an essential component of a number of dehydrogenases (Vallee *et al.*, 1956). Although there is no evidence yet on the location of such enzymes in *E. coli*, a study of enzyme localization in the related *B. megatherium* (Storck and Wachsman, 1957) has shown that a considerable variety of dehydrogenases are indeed located in the cell membrane. It may also be pertinent that, for one particular zinc enzyme, triose phosphate dehydrogenase (Racker, 1954), it has been found that thiolester bonds are formed and cleaved during the action of this enzyme. In any case, it appears that the phage particle utilizes some normal cellular component which contains zinc.

### 4. *Contraction of Proximal Tail Protein*

After T2 has interacted with cell walls, the tail fibers are completely removed and the particles appear to have a short, thick tail with the core protruding (Fig. 8—Kellenberger and Arber, 1955). Probably the main reason for the hesitation in believing that the proximal tail protein did actually shorten and thicken (i.e., contract) was that little evidence was available on how much of the tail structure of the intact particle was composed of the fibers. Lacking this information, it seemed not unlikely that the tail fibers might reach halfway up the tail (Kellenberger and Arber, 1955) and that the thickening might be an artifact of the electron micrograph. However, it is

now clear, from the work of Kellenberger and Séchaud (1955) on free tail cores, that these fibers comprise only the structure at the end of the tail, as illustrated diagrammatically in Fig. 6.

Evidence that the tail fibers comprise only the tip of the tail of the intact phage is provided by the electron micrographs of T2 treated by pH 10 glycine buffer (Kozloff, 1958). It can be seen in Fig. 10 that in the early stages of the action of the alkaline solution on the virus particle the tail fibers are unwound from the tip of the tail. The head loses its distinctive shape and, apparently, also its nucleic acid. After more prolonged treatment the head and the tail fibers are completely removed from most of the particles. However, even after the removal of the fibers, the tails are approximately their normal length and width. From most of the free tails there appears another structure, which probably is the tail core.

These results on the alkaline degradation of the virus particle clearly show, not only that the fibers comprise only the tail tip, but that under these conditions the proximal tail does not contract. Contraction does occur when the pH of the alkaline solution containing these free tails is subsequently lowered to 6.6 (Fig. 10c). This behavior is analogous to that of myosin, which contracts only when the pH is below 10, presumably because the Mg-myosin complex and adenosine triphosphate have the same charge at pH 10 (Morales, 1956).

The analogous behavior of the proximal tail protein and myosin led to an examination of other properties of the proximal tail that might be similar to those of myosin (Kozloff, 1958). Under certain conditions, EDTA (ethylenediaminetetraacetic acid) inhibits the contraction of actomyosin (Friess, 1954; Tonomura *et al.*, 1957). Furthermore, it has been shown that EDTA can cause a relaxation of contracted muscle fibers (Watanabe and Sleator, 1957). It has been found that sodium EDTA does not affect attachment of T2 to the intact host cell, but that it does prevent the formation of infective centers (Kozloff and Henderson, 1955) by preventing injection of DNA into the host cell (Kozloff, 1958). A similar effect was shown for the staphylococcal bacteriophages (Rountree, 1955). In this connection it is worth mentioning the experiments of Luria and Steiner (1954), which showed that there is a calcium requirement for the penetration of T5 DNA into the host cell. T5 has a long thin tail and it is possible that a high concentration of calcium is necessary for the contraction of the tail protein in order to allow the subsequent release of the DNA.

A strikingly analogous pattern of the properties of invasion mechanism of the phage tail and of actomyosin can be seen in Table IV. Various monovalent cations affect the inhibition of myosin ATPase (adenosine triphosphatase) by EDTA. In the presence of  $\text{Na}^+$  and  $\text{Li}^+$ , EDTA completely inhibits myosin ATPase. Substitution of  $\text{K}^+$  or  $\text{NH}_4^+$  for  $\text{Na}^+$  completely

reverses this inhibition and greatly stimulates the myosin ATPase. Similarly,  $K^+$  and  $NH_4^+$  allow the infection of the host cell by T2 in the presence of EDTA, although no infection occurs in the presence of  $Na^+$  or  $Li^+$ .

TABLE IV

EFFECT OF EDTA PLUS VARIOUS MONOVALENT CATIONS ON MYOSIN ATPASE ACTIVITY AND ON INFECTION OF *E. coli* B BY T2

| Agent            | Myosin ATPase <sup>a</sup> | T2 Infection <sup>b</sup> |
|------------------|----------------------------|---------------------------|
| No EDTA + $Na^+$ | 0.27                       | 250                       |
| EDTA + $Na^+$    | 0.00                       | 0                         |
| EDTA + $Li^+$    | 0.02                       | 0                         |
| EDTA + $K^+$     | 0.38                       | 50                        |
| EDTA + $NH_4^+$  | 0.79                       | 250                       |

<sup>a</sup> Taken from Kielley and associates (1956). The values are for the amount of inorganic phosphate liberated under their test conditions: 0.001 *M* EDTA, pH 7.7, monovalent cation 0.3 *M*.

<sup>b</sup> The values given represent the number of infective centers ( $\times 10^{-6}$ ) formed in the presence of EDTA (0.01 *M*) and 0.6 *M* salt (Kozloff, 1958).

The effect of sodium EDTA on the morphological alteration of the phage tail during interaction with the cell wall is shown in Fig. 10d. In this micrograph, the three particles that can be seen have lost their tail fibers, and the tail core has become visible. However, the degree of contraction in the presence of the sodium EDTA is markedly less than that observed when sodium EDTA is absent (see Fig. 8 and Fig. 10c). In the presence of sodium EDTA, the proximal tail protein contracts to 70 % of its length as compared to the 50 % contraction in the absence of sodium EDTA. The relative thicknesses of the proximal tail proteins is in agreement with these differences in shortening. It can be concluded that sodium EDTA inhibits the rate and degree of contraction of the proximal tail protein, similar to its action on actomyosin.

### *B. Action of Phage Enzyme on the Host Cell Wall*

When the phenomena of "lysis-from-without" was described by Delbrück (1940b), it seemed likely that the rapid lysis of susceptible cells by large numbers of phage particles was probably due to lytic activity in the phage particles. Anderson (1945) showed that a lytic substance could be released from phage by irradiation. Later, Weidel (1951) assumed that the decomposition of the bacterial cell wall which he observed was the result of the action



of an enzyme associated with the bacteriophage. The direct demonstration of an enzyme in T2 phage was shown by Barrington and Kozloff (1954, 1956), and confirmed by Koch and Weidel (1956).

Under conditions where up to 15 phage particles are adsorbed, each particle causes the liberation of the same amount of material. This implies that the enzyme can break down only a limited amount of substrate. It is of some interest to note that the amount of cell wall nitrogen released by one phage particle is about  $5 \times 10^{-14}$  mg., which is similar to the amount of nitrogen in the phage DNA.

The phage enzyme apparently is not acting upon the attachment site on the cell. Jesaitis and Goebel (1955) and Weidel and Kellenberger (1955) have shown that the isolated attachment sites for T4 and T5 (Fig. 6) are not broken down by interaction with a phage. Although the precise nature of the substrate for the enzyme is not known, Weidel and Pringosigh (1957) have shown that the cell wall material released by large excess of phage enzyme comprises most of the structural units of the cell wall which give it its rigidity. The enzyme apparently does not affect the cell membrane.

Shortly after attachment of a phage particle, there is a temporary change in cell permeability which allows some of the cell contents to leak out of the cell (Puck and Lee, 1954, 1955). Since 0.25 *M*  $Mg^{++}$  or  $Ca^{++}$  inhibits both leakage (Puck and Lee, 1955) and the action of the phage enzyme on the cell wall (Brown and Kozloff, 1957), the leakage probably is due to the action of the phage enzyme on the outer cell wall. However, there is some confusion about the relationship of the leakage due to the action of phage enzyme and that due to the effects of other agents which also cause cell lysis (Tolmach, 1957). Any agent which interferes with the normal synthesis of the cell wall components also leads to leakage and lysis by reactions unrelated to those caused by the phage particle (Prestidge and Pardee, 1957). The fact that leakage stops within 3–5 minutes after infection is probably a feature of normal metabolism which maintains the cell wall integrity.

Studies on the morphological localization of phage enzyme, which followed closely after the work on the structure of the phage tail, revealed that the intact phage is enzymatically inactive (Brown and Kozloff, 1957; Kozloff and Lute, 1957a) and that the enzyme is exposed only after the tail is altered by removal of the tail fibers. Although it would be desirable to isolate and test the enzymatic activity of tail cores in order to be certain that they are inactive ( $H_2O_2$ -treated phage have tail cores and are inactive), at the present time it seems likely that the enzyme is located on the proximal tail protein. It should be repeated in this connection that the proximal tail protein apparently contracts upon interacting with the cell wall. The question can be raised whether contraction is necessary to expose the enzyme and, further, what is the relation between the phage enzyme and myosin-like



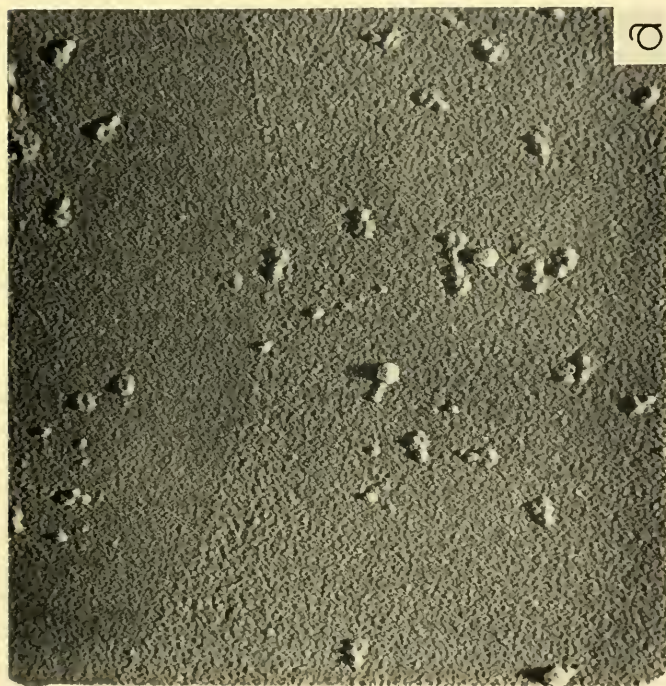
proximal tail protein. Since sodium EDTA does not affect the action of intact phage on cell walls (Barrington and Kozloff, 1956; Kozloff and Lute, 1957a), although it inhibits the contraction of the proximal tail proteins, it seems likely that the enzyme is immediately exposed after the removal of the tail fibers or after only a slight contraction, and is probably located close to the distal end of the uncontracted proximal tail. Although the viral enzyme has not been isolated and has been only partially characterized, the occurrence of typical phage enzyme activity in a nonsedimentable form in lysates (Brown, 1956; Koch and Jordan, 1957) suggests that the enzyme is synthesized quite independently of the proximal tail protein. Koch and Jordan (1957) have claimed that the phage enzyme is relatively small, having a molecular weight of about 20,000. This would also differentiate it from the relatively large particulate proximal tail protein.

It is difficult to ascribe to any one particular reaction between the phage and the cell the phenomena of irreversible attachment and cell death (see Sections II and V). Once the virus has been altered the attachment is irreversible, but irreversible attachment occurs even with zinc-deficient cell walls (Kozloff and Lute, 1957a) where there can be little or no removal of phage tail fibers. Presumably, an irreversible bond between the phage and the cell wall is formed before the tail fibers are split off. In fact, the firm binding of the tail fibers might play an important role in holding the phage particle on the cell wall after the fibers have been unwound from the tail core. This would serve to keep the enzyme in close contact with its substrate.

### *C. Release of Phage DNA*

The factors controlling the release of phage DNA from its protein covering and the passage of the DNA into the cell are poorly understood. Some of the physical forces which might affect the passage of DNA into the cell have been discussed recently in a theoretical paper by Ore and Pollard (1956). It was shown that ordinary forces, such as linear Brownian movement, and the centrifugal pull exerted by oscillatory thermal movements, could largely account for the rate of DNA injection. However, no consideration was given to the changes which occur in the viral tail structure or to the effect of electrostatic interaction between the phage DNA and protein. Both of these factors would be expected to influence greatly the rate of DNA release.

Part of the difficulty in studying the injection mechanism has been due to the difficulty in isolating experimentally this phase of the process from other reactions. Recently, T2, altered by treatment with cadmium cyanide complexes in a manner presumably identical to that produced by interaction with the cell wall, has been used to study the factors affecting the release of DNA (Kozloff and Lute, 1957b). After alteration the tail is 50 % shorter and it can be estimated that the central hole in the tail (after the removal of the



a



b

FIG. 11. Release of DNA from T2; (a) T2 treated first with  $\text{Cd}(\text{CN})_3^-$  and then with  $0.1\text{ }M$  lysine. Note that all the altered particles appear to have lost their DNA (Magnification  $\times 29,000$ ); (b) T2 treated first with  $\text{Cd}(\text{CN})_3^-$  and then with  $0.02\text{ }M$  lysine (Magnification about  $\times 29,000$ ).



core) has been widened from about 100 to 140 Å. Since the viscous drag in passing a solution containing a highly asymmetrical molecule such as DNA (diameter about 25 Å) through a narrow tube is directly proportional to the length and inversely proportional to the fourth power of the diameter of the tube, these morphological changes reduce the viscosity barrier, i.e. the flow time, by about 8-fold. The properties of T2 altered by  $\text{Cd}(\text{CN})_3^-$  or by cell walls support the concept that viscosity is at least one barrier to DNA release. In saline, altered phage do not measurably release their DNA at 10–15°C.; at 37°C. the DNA is very slowly released, but at 44°C. the DNA is released very rapidly. It is well known that an increase in temperature greatly lowers the viscosity of DNA solutions.

Since the injection of phage DNA into the host cell occurs rapidly at 37°C., special devices would seem necessary to overcome, not only the viscosity barrier, but also the attractive forces between the DNA and phage protein. It has been found, for instance, that at neutral pH's free phage DNA and protein spontaneously aggregate (Kozloff and Lute, 1957b). In considering the many steps in the invasion mechanism where one reaction controls or initiates the succeeding reaction, it might be expected that DNA release would be chemically controlled and that some compounds would initiate DNA release. In the model experiments using  $\text{Cd}(\text{CN})_3^-$ -altered T2, it was found that various compounds containing primary amino groups, such as glucosamine, and various amino acids, such as lysine, caused the rapid release of the phage DNA from its protein covering. The release occurred optimally at pH 8.75–9.0 and appeared to be an all-or-none phenomenon (Fig. 11). It is attractive to consider the action of the various compounds which cause the DNA release as being analogous to the action of similar agents produced during invasion. Almost all of the compounds which cause DNA release are found in the cell wall. It can be suggested that the products formed by the action of the phage tail enzyme on the host cell wall might be the agents which trigger the release of the DNA into the host cell. It is worth noting that the products of cell wall digestion (Weidel and Pringosigh, 1957) have free amino groups and include glucosamine, one of the most active agents in causing DNA release from altered phage, as well as muramic acid, diaminopimelic acid, glutamic acid, and alanine.

#### *D. Summary of the Steps of Invasion*

The sequence of reactions that appear to be involved in invasion by T2 is summarized diagrammatically in Fig. 12. It is perhaps unnecessary to point out that the evidence is not equally strong for each step. This summary may serve as a guide to further experimentation which undoubtedly will modify the present views on the subject.



### 1. Attachment

The phage attaches by its tail to the host cell. The initial attachment reaction occurs between a specific cell wall receptor and the tail fibers of the phage, and may be reversible. Almost immediately after the initial reaction a more permanent bond of unknown nature is formed.

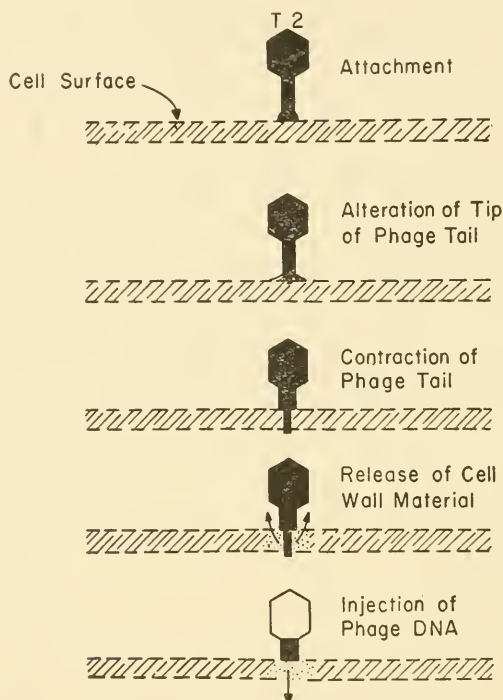


FIG. 12. Diagrammatic representation of the reactions involved in T2 invasion.

### 2. Alteration of the Tip of the Phage Tail

There is strong evidence that the fibers at the tip of the tail are unwound and eventually removed after permanent attachment to the cell wall. Zinc, tightly bound to cell wall protein, is essential for the unwinding and removal of the tail fibers. The tail fibers appear to be attached to each other (and to the tail core) by thiolester bonds. It is probably during this stage of invasion that the tail enzyme, which hydrolyzes cell wall material, is exposed.

### 3. Contraction of the Phage Tail

The evidence for contraction is based on the morphological changes which occur under various conditions and on the striking parallelism of the invasion



process with several properties of actomyosin. At present it would be unwarranted to label the proximal tail protein as a form of actomyosin, especially in view of lack of information about the behavior of the tail protein toward ATP. The exact function of the contractile step is not yet apparent. It might be proposed that the contraction of the proximal protein aids in penetrating the cell wall by forcing the tail core through the cell membrane. The tail core may then come out of the tail and perhaps remain inside the cell. On the other hand, the contractile step may be more like the opening of a sphincter to permit the DNA to pass through the tail.

#### 4. Release of Cell Wall Material

An enzyme in the phage tail removes a part of the cell wall that is responsible for its rigidity. This enzyme apparently acts only on the limited area of the cell wall which is in contact with the enzyme. Possibly the contractile step ensures close contact of the insoluble enzyme with the insoluble substrate.

#### 5. Injection of Phage DNA

The forces involved in injecting the phage DNA are not known. The decrease in the viscosity barrier when the proximal tail protein contracts does not permit the immediate release of the DNA. A chemical triggering agent would seem to be necessary. In model experiments DNA is released from the head by a variety of compounds which contain primary amino groups. The best candidate for this role during invasion is the material released by the action of the tail enzyme on the cell wall, since it contains free amino groups.

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## Chapter VII

### Intracellular Multiplication of Bacterial Viruses

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*La bactériologie n'a jusqu'ici envisagé que le 'problème des deux corps,' bactérie et milieu, que ce milieu soit l'organisme parasité ou le liquide au sein duquel se fait la culture, et ce problème était déjà bien complexe; il l'était pourtant beaucoup moins que le 'problème des trois corps' que nous allons aborder, où nous aurons à envisager les interactions entre le milieu, milieu de culture ou organisme parasité, la bactérie parasitant ce milieu et l'ultramicrobe bacteriophage parasite dans la bactérie. d'Hérelle (1921).*

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## I. INTRODUCTION

Within a few years of Twort's (1915) and his own first description (d'Hérelle, 1917) of a serially transmissible bacteriolytic principle, d'Hérelle had understood the fundamental nature of the multiplication of bacterial viruses. In 1926, d'Hérelle thus summarized his earlier findings: "The first act of bacteriophagy consists in the approach of the bacteriophage corpuscle toward the bacteria, then in the fixation of the corpuscle to the latter. . . . The bacteriophage corpuscle penetrates into the interior of the bacterial cell. When, as a result of its faculty of multiplication, the bacteriophage corpuscle which has penetrated into the bacterium forms a colony of a number of elements, the bacterium ruptures suddenly, liberating into the medium young corpuscles which are then ready to continue the action (d'Hérelle, 1920, 1921)." Far from gaining immediate acceptance, however, d'Hérelle's views found few followers among bacteriologists, "probably," wrote Burnet in 1934, "because of the heterodox nature of these conceptions rather than from any fault in d'Hérelle's logic." Some of the early investigators of the bacteriophage, such as Kabeshima (1920), and Bordet and Ciuca (1920), denied in fact the existence of any phage "corpuscles" and believed that the only important feature of bacteriophagy was the induction of bacterial lysis, a phenomenon which they fancied to be caused by enzymes either secreted by an animal host or endogenous to the bacteria themselves. Hence, from this standpoint, the question of bacterial virus *multiplication* is not meaningful, the only real problem being the mechanism of induction of lysis of the bacterial cells. Other workers, while more or less accepting d'Hérelle's notions of the self-reproducing nature of the bacteriophage, thought, on the contrary, that "far from being the product of lysis (the phage) is regenerated during the stage of active multiplication of susceptible bacteria preceding the lysis. . . . Lysis of the bacteria is merely a secondary phenomenon which may or may not follow the accumulation of phage" (Bronfenbrenner, 1928). Krueger and Northrop (1930) imagined, in fact that the bacteriophage freely passes into and out of the bacterial cells, so that extra- and intracellular phage particles exist in a state of equilibrium with one another.

For twenty years, "this controversy persisted with gradually diminishing intensity but without the appearance of any unanimity," although "however agnostic they may have been in regard to the nature of a phage, all workers manipulated and in practice thought of it as an extrinsic virus-like agent" (Burnet, 1934). The controversy finally subsided in the 1940's, when a new generation of investigators took up the study of the mechanism of bacterial virus multiplication more or less *de novo*. Most of the participants in this latter-day renaissance of bacteriophage research confined their attention to the study of one and the same group of bacterial viruses, the seven "T" phages active on *Escherichia coli* (Demerec and Fano, 1945), and, in particular, to

the "T-even" strain T2, T4, and T6. This limited range of experimental material permitted direct comparison and integration of the results of many different workers and was of great heuristic value, although it now seems likely that the discovery and understanding of other aspects of bacteriophages of no less fundamental interest than their multiplication, e.g., of lysogeny and transduction, was retarded by the choice of this particular phage-host system. Almost all of the insight into the subject matter of the present chapter, however, has come from experiments carried out with the T-even phages. Hence, it may be assumed that, unless explicitly stated otherwise, and with the exception of the section devoted to comparative bacterial virology, the post-1940 work mentioned in the following pages concerns one of these three closely related bacteriophage strains.

The topics of this chapter have been the subject of numerous reviews since d'Hérelle's first monograph on the bacteriophage in 1921 and the publication of the polemics of d'Hérelle, Twort, Bordet, Gratia, and others at the 1922 Meeting of the British Medical Association (*Brit. Med. J.* **1922**, 289-299). The reader who wishes to acquire a first-hand acquaintance with the evolution of the knowledge of bacterial virus multiplication may profit by perusal of some of these reviews, like those of Bronfenbrenner (1928), Bordet (1931), Burnet (1934), Delbrück (1942, 1946), Cohen (1949), Luria (1950), and Hershey (1952, 1956).

## II. KINETICS OF PHAGE REPRODUCTION<sup>1</sup>

### *A. One-Step Growth*

The basic manifestation of bacteriophagy is the following: A growing bacterial culture is inoculated with an emulsified sample of the ecological environment of the bacterial type under study, whereupon lysis of the infected culture suddenly ensues several hours later. A second bacterial culture is now infected with a very high dilution of this lysate and lysis of the second culture again ensues suddenly after several hours' incubation. The process of lysing bacterial cultures by inoculation with high dilutions of previous lysates can be continued indefinitely, i.e., the lytic principle, or bacteriophage, is serially transmissible. From the very start, d'Hérelle interpreted this phenomenon in terms of infection of a few bacteria of the culture by a few phage particles in the inoculum, followed by intrabacterial multiplication of the infecting phages to yield an issue of many progeny

<sup>1</sup> In all of the experiments discussed in this chapter, the assay for the number of infective bacteriophage particles is carried out by means of the *plaque-counting method* and the temperature of incubation is in the vicinity of 37°C. A description of the experimental techniques and methods employed in modern bacteriophage research can be found in Adams (1950).

particles, release of the progeny by lysis of the infected cells, reinfection of a much greater number of bacteria, and repetition of the intrabacterial multiplication-lysis-reinfection cycle until a sufficient number of phage particles have been produced so that all of the cells of the culture are infected and finally lysed. Although this idea now appears simple enough, it did not seem to be clearly understood by d'Hérelle's critics. Bordet, for instance, remarked at the occasion of his Croonian Lecture (Bordet, 1931): "It would be difficult to suppose that an intrabacterial virus, originally present in small amount and at first allowing the microbes to develop, attacks them all at a given moment, almost simultaneously." D'Hérelle, however, had some experimental support for his view, since he could show that the multiplication of the bacteriophages from a small inoculum of a few particles to the final yield of several million progeny proceeds in a *stepwise* manner in the culture, each step requiring about 75 minutes and leading to an approximately 20-fold increase in the total concentration of phage particles. Burnet (1929) was able to adduce more convincing proof concerning the stepwise nature of phage multiplication by an improved experimental arrangement. He placed a sufficiently small aliquot of a phage suspension into each of a large number of tubes containing a small volume of a growing bacterial culture, so that each tube received, on the average, one or two phage particles. This set of tubes was then incubated and the total content of a number of single tubes plated for assay at one-minute intervals on agar plates seeded with sensitive indicator bacteria. The result of this experiment, the progenitor of the present day "single-burst" technique, was that for the first 20 minutes after the bacteria are first infected, each tube contains either none or only one or two plaque-forming infective centers and that after more than 20 minutes have elapsed, many of the tubes suddenly contain anywhere from 20 to 100 infective centers. Burnet concluded from this result that: "the phage particle has clearly for the first 20 minutes been multiplying under some spatial constraint which is then suddenly released. When we combine this information with the fact that when a large excess of phage is added to multiplying bacteria in broth or in agar, visible lysis as judged microscopically or macroscopically occurs about 20 minutes after the addition, one must admit that the liberation from spatial constraint can only be synonymous with the disruption of the invaded bacterium and the associated liberation of (progeny) phage particles into the medium."

In 1939, Ellis and Delbrück devised the *one-step growth* experiment, which clearly demonstrated that the progeny of the infecting bacteriophage particle appear only after a period of constant virus titer and provided also the proof that no further production of bacteriophages takes place if reinfection by phage progeny of any uninfected bacteria in the culture is prevented. The one-step growth experiment, which today represents the basic procedure for

studying bacteriophage multiplication, consists of infecting a growing suspension of bacteria with a suitable number of phages, incubating this mixture for a few minutes in order to allow adsorption to the bacteria of most of the phage particles, and then diluting this mixture from ten thousand- to a millionfold into nutrient medium. The diluted culture is then incubated further and aliquots plated on sensitive indicator bacteria at regular time intervals in order to assay the instantaneous number of infective centers in the culture. The result of Ellis and Delbrück's one-step growth experiment was that the number of infective centers in the culture remains constant for the first 30 minutes after infection of the bacterial culture. This initial time interval, during which the number of infective centers shows no increase, is the *latent period*. After 30 minutes have elapsed, the number of infective centers in the culture begins to augment until a final plateau is attained 50 minutes later, when no further increase in infectivity occurs; the time interval during which the number of infective centers increases is the *rise period* and the ratio of the titer represented by final plateau to the initial titer is the *burst size*, which in Ellis and Delbrück's case was approximately 60. The latent period thus represents the minimum time which must elapse between the moment at which the bacterial culture is infected with a phage population and the moment at which the first infected cells in the culture lyse to liberate into the medium the progeny phage particle which have grown within them. The rise period represents the interval during which more and more of the infected bacteria lyse, and the final plateau represents the stage at which all of the infected bacteria have lysed and no further phage multiplication occurs, progeny phage and any remaining uninfected bacteria in the culture having been separated from one another in the growth tube by the high dilution made shortly after the outset of the experiment. The burst size, finally, represents the average number of infective progeny phage particles produced per infected bacterium.

One of the few later improvements in the one-step growth technique has been an induction of more synchronous phage growth by adsorbing the infecting phage particles to starved bacteria in a non-nutrient medium and then declenching phage development simultaneously in all bacteria by addition of nutrient (Benzer, 1952). Adsorption of phage to bacteria suspended in growth media containing cyanide (Benzer and Jacob, 1953) or chloramphenicol (Tomizawa and Sunakawa, 1956; Hershey and Melechen, 1957) and subsequent initiation of phage development by removal of the drug can achieve similar results. These procedures eliminate that variation in the onset of intracellular phage growth which is due to the spread in times at which different bacteria adsorb their first phage particle.

The parameters of phage multiplication defined by the one-step growth experiment may differ very much, according to the exact physiological



conditions under which growth is allowed to proceed. The latent period, not surprisingly, is longer at lower incubation temperatures. Ellis and Delbrück (1939) found, for instance, that the latent period of their coliphage is 30 minutes at 37°, 60 minutes at 25°, and 180 minutes at 16°C. Maaløe (1950), who examined in more detail the prolongation of the latent period at lower temperatures, observed that the temperature effect is not the same at different points in the latent period, as if the successive stages of intracellular phage multiplication possess very different temperature sensitivities. The nutrition and physiological "age" of the bacterial host cell can also affect both latent period and burst size. In a poor growth medium or in lag-phase cultures the latent period is often longer and the burst size smaller than in bacteria growing rapidly on a favorable substrate (Delbrück, 1940; Héden, 1951). Latent period and burst size, finally, differ widely for various types of bacteriophages (cf. Delbrück, 1946) and may vary even for different mutants of the same phage strain (Symonds, 1957), or for the same phage strain growing on different hosts (Barry and Goebel, 1951). The latent period, however, does not appear to depend in any very striking way on the *number of phage particles* with which each bacterial cell has been infected, since the first progeny phages make their appearance at about the same time, whether the concentration of infecting phages is much less than that of the host cells (in which case each bacterium which is infected at all is infected with only a single particle), or whether the concentration of infecting phages greatly exceeds that of the host cells (in which case each bacterium is infected with many particles) (Ellis and Delbrück, 1939; Delbrück, 1940).

### B. Single Burst

The one-step growth experiment follows the mass behavior of thousands of phage-infected bacteria. The latent period, therefore, is a *minimum* parameter and reflects only the time required for lysis of those cells in which phage development has progressed most rapidly, while the burst size reflects only the *average* phage yield per cell. Ellis and Delbrück (1939) also devised a *single-burst experiment*, by means of which it is possible to study phage development in individual infected bacteria. Immediately after infection, numerous small aliquots of a high dilution of the culture of a one-step growth experiment are placed into separate little tubes, so that each tube contains on the average less than one infected bacterium. If the average number of infected bacteria per tube is  $m$ , then the fraction of tubes,  $p_r$ , having received  $r$  infected cells is given by the Poisson law

$$p_r = \frac{m^r}{r!} e^{-m} \quad (1)$$

Hence, provided that  $m$  is sufficiently less than one, most of those tubes which have any infected bacteria will have only one. The tubes are then incubated



until lysis of all the infected bacteria is certain to have occurred and the total contents of each tube plated for assay of the number of infective progeny. In the experiment of Ellis and Delbrück, 25 out of 40 tubes, or a fraction of 0.62, showed no plaques upon plating, i.e., did not contain any infected bacteria at all. The remaining 15 tubes showed anywhere from 5 to 190 plaques, of which, according to (1), about 12 plates probably represent the yield from a single infected bacteria. The burst sizes of individual infected cells are thus seen to be subject to considerable variations. More extensive experiments by Delbrück (1945a) showed these variations to be extremely wide indeed, since rare bacterial cells were observed which liberated more than 1000 progeny phages. An average burst size may also be calculated from the single-burst data by dividing the total number of plaques on all plates by the number of infected bacteria which have given rise to the entire progeny brood. This ratio is usually found to be similar to the average burst size estimated directly from a one-step growth experiment.

The reasons underlying the very broad distribution of individual burst sizes still remain obscure. Delbrück (1945a) noted that the variation in length of individual bacteria of the infected culture was very much less than the variation in number of progeny particles which these cells liberate. Delbrück inferred, therefore, that variable cell size was probably not the factor involved in burst size variation. It seems possible that it is the *complexity* and, in part, geometric nature of the process of intracellular phage growth itself which is responsible for the wide fluctuations in phage yields of individual infected cells. For if the final number of mature progeny phage particles liberated at the time of lysis depends on a number of consecutive as well as concurrent reaction steps, then relatively small perturbations in any one of these reactions can easily lead to a very considerable diminution of the maximum number of phages of whose synthesis the cell is capable under very optimal conditions.

### *C. Lysis and Lysis Inhibition*

The kinetics of bacteriophage growth may be followed not only by assaying the infective titer in one-step growth experiments, but also by observation of the lysis of the infected cells, either through direct microscopic examination of the bacterial cells or through macroscopic measurement of the turbidity of the bacterial culture. In such experiments it is necessary, of course, that nearly all of the bacteria are infected at the outset of the experiment, i.e., that the average number of phage particles adsorbed per bacterium, or the multiplicity of infection, is greater than one. By following the time course of lysis in a typical one-step growth experiment, it can be seen that the number of intact bacteria visible under the microscope and the turbidity of the infected culture begin to decrease precisely at the conclusion of the latent

period and reach their minimum value when the final plateau of progeny infectivity has been attained, i.e., when all the bacteria which are going to lyse have lysed (Delbrück, 1940; Doermann, 1952).

The mechanism by which the phage-infected bacterial cell is lysed has been the object of numerous investigations since the first days of bacteriophage research. Nevertheless, the nature of the lytic reaction still does not appear to be entirely understood. Some of the early observations were bedeviled by the circumstance that there exists not one but two completely different processes by which bacteriophages can lyse susceptible bacterial cells (Delbrück, 1940). One of these, *lysis-from-without*, represents an immediate dissolution of bacteria, often encountered when the multiplicity of infection is much greater than one (Bail and Matsumoto, 1923; Meuli, 1923; Krueger and Northrop, 1930). Loss of the input phages, rather than their multiplication, is connected with this form of lysis, which can proceed in, or is even favoured by, the absence of an active metabolism of the host cell (Heagy, 1950). It seems very likely that lysis-from-without is a consequence of the digestion of parts of the bacterial cell wall by the attachment organs of the adsorbed phage particles (Weidel, 1951; Puck, 1953; Barrington and Kozloff, 1956; cf. Chapter 2). Since in the course of intracellular phage growth, bacteria become progressively more resistant to lysis-from-without induced by subsequent superinfection with a high multiplicity of secondary phage particles (Visconti, 1953), it can only be the second of the lytic processes, or *lysis-from-within*, that is really the form of lysis properly connected with intracellular phage multiplication and hence of concern to us here. Direct microscopic observations of the lysis of phage-infected, rod-shaped bacteria were already carried out by d'Hérelle (1921), and later by daCosta Cruz (1926), who noticed that the cells assume a swollen or spherical form shortly before their dissolution. Use of microcinematography showed that the dissolution of the bacteria and the release of their contents resembles more an explosion, as if the final act of cell lysis corresponds to a bursting of the cell envelope by internal pressure (Bronfenbrenner, 1928; Bayne-Jones and Sandholzer, 1933). Although it may be the internal pressure which delivers the *coup de grâce* to the infected cell, it is probable that intracellular phage multiplication first engenders a weakening of the structural members of the bacterium, so that the rod-shaped cell loses its rigidity and, shortly before its end, assumes the spherical shape of least surface-to-volume ratio, before the tensile strength of the envelope falls below the stress exerted by the osmotic forces.

The weakening of the bacterial structures connected with lysis-from-within has generally been thought to be the work of intrabacterial enzymes, although opinions have differed as to whether the lytic enzymes are the "endoferments" responsible also for the autolysis of uninfected bacteria (Bronfenbrenner, 1928; Wollman and Wollman, 1933), or whether they

represent a special type of enzyme whose synthesis is induced specifically by the infecting bacteriophage. Many of these opinions are based on the presence in phage lysates of *lysins*, or agents which are distinct from the phage particles and which are themselves capable of inducing lysis of uninfected, heat-killed bacteria (Sertic, 1929; Schuurman, 1936; Gratia, 1937; Panijel and Huppert, 1954; Maxted, 1957; Ralston *et al.*, 1957). Since by virtue of the methods used in their detection, however, the action of these lysins must necessarily resemble lysis-from-without, it has still not been shown what role, if any, lysins play in lysis-from-within. Inasmuch as fragments of the intact phage particle can also induce lysis-from-without (Herriott, 1951; Herriott and Barlow, 1957), it is quite possible that some, or all, of the lysins are *incomplete* bacteriophages instead of the intracellular lytic enzymes (cf. Section III, A). The fact that a high concentration of lysin is produced after development of certain "defective" bacteriophages supports this view (Jacob *et al.*, 1957). It is not even clear what could be the substrate for the intracellular lytic enzymes, since bacteriophage growth and lysis-from-within proceed more or less normally in bacterial protoplasts, from which the cell wall, *a priori* the most likely substrate and that most certainly involved in lysis-from-without, has already been removed (Brenner and Stent, 1955; Mutsaers, 1955; Salton and McQuillen, 1955). In any case, the initiation of the reactions responsible for lysis-from-within appears to require not only infection of the bacterium but also onset of intracellular phage development. This may be inferred from the fact that bacteria do not lyse which have been infected with nonmultiplying virus particles, such as ultraviolet-irradiated, or certain types of mutant and host-modified bacteriophages (Luria and Human, 1950; Benzer, 1955; Luria, 1953; Bertani and Weigle, 1953). The formation of infective progeny, on the other hand, does not seem to be prerequisite for lysis-from-within, for in the presence of the dye proflavine infected bacteria lyse at the normal time without yielding any phage (cf. Section III, C, 2). In the course of normal phage growth, the lytic reaction seems to be initiated about halfway through the latent period, since after that time a rapid degeneration of the host cell sets in which leads to its lysis a few minutes later, even if further phage multiplication is arrested by cooling to low temperatures or by addition of such poisons as cyanide (Maaløe, 1950; Bentzon *et al.*, 1951).

There exists, however, a "natural" way in which the imminent disintegration of a phage-infected cell can be staved off at almost the last moment. Doermann (1948a) discovered that if bacteria infected with one of the T-even phages are *superinfected* with additional phage particles shortly before the end of the normal latent period, then the appearance of progeny phages delayed by several minutes and the over-all lysis of the culture, as reflected by loss of its turbidity, is retarded by as much as an hour. This is the phenomenon of *lysis inhibition*. When lysis-inhibited bacteria finally do lyse,

they yield a very high burst of progeny phage particles, which can sometimes attain an average of more than a thousand per cell. "Rapid lysis," or  $r$ , mutants of the T-even strains occur, however, which do not produce lysis inhibition, in that bacteria primarily infected with such  $r$  mutants lyse at the normal time, whether or not superinfected with additional phage particles prior to the end of the latent period, even if the superinfecting particles are of the wild  $r^+$ , or lysis-inhibiting type (Doermann, 1948a). Bacteria primarily infected with  $r^+$  phages, however, will manifest lysis inhibition even if superinfected with rapid lysis  $r$  mutants (Stent and Maaløe, 1953). One may infer, therefore, that the intracellular growth of the  $r^+$  wild type bacteriophage causes the host cell to degenerate in a way different from that engendered by the growth of the  $r$  mutants, so that in the former case the adsorption of  $r^+$  or  $r$  particles at late stages of the latent period strengthens the cell, or at least retards further degeneration, thus postponing the moment of ultimate disruption by the internal pressure. Lysis inhibition, although its mechanism is still poorly understood, has been an extremely useful tool in both genetic and biochemical studies of bacteriophage reproduction, since it allows harvest of additional infective progeny particles constituted from bacteriophage substance already present in an "incomplete" form at the time of normal lysis (Levinthal and Visconti, 1953; Stent and Maaløe, 1953).

#### *D. The Eclipse*

Although the one-step growth experiment demonstrated clearly the general nature of the process by which bacterial viruses multiply within cultures of susceptible bacteria, it only brought into focus rather than answered the question of more fundamental biological interest, namely, what is actually going on inside of the infected cell during the latent period while the parental phage particle manages to cause its own several hundredfold reduplication. One important point relevant to this question is naturally the manner in which the number of phage particles present *within* the infected cell increases from the moment of infection until the time of lysis. This information can be obtained by breaking open the infected cells at various times during the latent period and assaying the infectivity of the material released by premature lysis. Such an experiment was undertaken by Doermann (1948b, 1952), who infected bacteria with T4 phages under the conditions of the one-step growth experiment and induced lysis-from-without of aliquots of the infected culture at various times after infection by addition of cyanide and a large excess of T6 phages. Doermann then assayed these artificial lysates for their content of infective T4 particles by use of indicator bacteria resistant to the action of the T6 employed for lysis-from-without. The result of Doermann's experiment was that *the infectivity associated with the original*



*parental bacteriophages is lost at the outset of the reproductive process*, since no infective particles whatsoever can be found in any of the lysates in which the infected bacteria have been opened within 10 minutes following infection. After more than ten minutes have elapsed, however, an ever-increasing number of infective particles make their intracellular appearance until the final crop of progeny has been attained which would have been released by the spontaneous lysis of all the infected bacteria at the end of the normal latent and rise periods. The time intervening between infection and first intracellular appearance of infective progeny particles, i.e., the stage of intracellular bacterial virus growth during which the infected host cell contains no material capable of infecting another bacterium, is called the *eclipse* (Luria, 1950). The discovery of the eclipse period confirmed an earlier observation of Wollman and Wollman (1937), who opened up phage-infected *Megatherium* bacilli by lysozyme digestion of the cell wall and noticed that the infectivity associated with the parental phage particles could not be recovered from the artificial lysate. The Wollmans already inferred from their finding that the infecting bacteriophage enters a noninfective, or "cryptophagic," phase in the course of its intracellular growth.

Other methods for premature lysis of phage-infected bacteria have been devised since Doermann's experiment. Among these may be mentioned *sonic oscillation* (Anderson and Doermann, 1952), *explosive decompression* through sudden release from a high-pressure nitrous oxide bomb (Fraser, 1951), and treatment of the phage-infected bacteria with either *glycine* (Kay, 1952; DeMars, 1955), *chloroform* (Séchaud and Kellenberger, 1956), or *streptomycin* (Symonds, 1957). In the case of phage-infected bacterial protoplasts, whose cell wall has already been digested away by enzyme treatment, immediate lysis can be induced at any moment by changing the suspending medium of the protoplasts from one of high to one of low osmotic pressure (Brenner and Stent, 1955; Salton and McQuillen, 1955). All these methods reveal exactly the same sequence of events of intracellular phage growth, i.e., disappearance of the infectivity of the parental phage particle, appearance within the cells of infective progeny particles after an eclipse lasting for about half of the latent period, and increase in the number of intracellular progeny at a *constant* rate. Such linear kinetics of appearance of infective progeny naturally represent only an average over the entire infected culture, and it was possible that in individual infected cells the increase in intracellular phages follows rather different, e.g., exponential, kinetics. In order to study the time course of phage growth in individual infected bacteria, therefore, the techniques of premature lysis and single burst have been combined. That is, a very large number of tubes, each containing less than one infected bacterium, were incubated and premature lysis induced at various times after infection. This experiment showed that the moment at



which individual bacteria contain their first infective progeny is distributed over many minutes and that after the termination of the eclipse, the infected bacteria pass through phases in which they first contain few and then many progeny phages, the rate of increase of such phages in individual cells indeed being constant (Bentzon *et al.*, 1951; Doermann, 1951).

The discovery of the eclipse period temporarily complicated the conclusions which one might have hoped to draw from Doermann's intracellular growth curve, for the question now posed itself as to just when it is during the latent period that the actual multiplication of the infecting phage particle takes place. It was possible that the eclipse period represents only a waiting stage for the infecting phage particle, during which it is "masked" while the host cell undergoes some necessary renovations preliminary to the onset of phage multiplication. In this case, the first infective phage particle present within the infected cell at the termination of the eclipse would be the original, once more "unmasked" parental phage, which then proceeds to grow and divide as does any other microbe (Fulton, 1953). The daughter viruses of the first division would likewise divide until the final crop of several hundred progeny viruses had been attained by the end of the latent period. One might expect that such geometric mode of increase of intracellular infective progeny should follow exponential rather than, as actually found, linear kinetics, unless limiting conditions imposed by the fact that the bacterial cell is a *bounded system* have already been attained by the end of the eclipse period.

An alternative explanation of the significance of the eclipse period was that, far from being a stage of waiting, the eclipse is that part of the latent period during which there occurs synthesis of the substance of the bacteriophage progeny. The increase in intracellular infective progeny in that case does not really represent the multiplication of the parental virus at all but constitutes, rather, a terminal process of *maturation* of previously synthesized progeny structures into intact virus particles. Genetic studies described in Chapter 5, as well as the experiments to be recounted now, have established that it is this latter point of view which is correct, i.e., that the parental bacteriophage particle not only metamorphoses into a noninfective form at the outset of its infection of the host cell, but that it also multiplies in this form to yield noninfective progeny structures whose maturation into infective viruses signals the end of the eclipse. This noninfective form is called the *vegetative phage*, in contradistinction to the *resting phage* represented by the mature infective virus particle (Hershey, 1952; Doermann, 1953). The vegetative phage is thus the connecting link between parental and progeny viruses, and the elucidation of its structure and function the central problem of phage growth.

### III. SYNTHESIS OF THE BACTERIOPHAGE PROGENY SUBSTANCE

*In a well-ordered kitchen, the basic cooking is done before the first course is served.*  
Luria (1950).

#### *A. Incomplete Bacteriophages*

The premature lysis experiment of Doermann followed the intracellular growth of the bacteriophage by assay of the intracellular number of infective phages, i.e., of progeny structures already endowed with the power of self-reproduction, the most complex of all the properties of the virus particle. Further insight into the process of phage multiplication was gained by the discovery of a variety of "incomplete" phage structures in premature lysates of phage-infected bacterial cultures. These structures are incomplete in the sense that they possess one or another of the properties of the virus without being endowed with the ability to cause plaques when plated for assay on a plate seeded with sensitive indicator bacteria. Two possible interpretations offer themselves as to the nature of the incomplete bacteriophages. They could, on one hand, be *phage precursors*, i.e., represent developmental stages in the ontogeny of the virus particle. In order to qualify for consideration as a phage precursor, any given type of incomplete structure should appear before the mature phage particles and later be incorporated into them in the subsequent course of intracellular growth. Some of the incomplete structures, on the other hand, could also represent *by-products* of phage reproduction or abortive attempts to construct intact phages, in which case they would not be destined to form part of the future infective progeny.

#### *1. Sedimentation, Adsorption, and Precipitation*

Three properties which set bacteriophages apart from other structures present in the host bacterium are their *sedimentability*, their *adsorbability*, and their *antiserum precipitability*. Because of their size, shape, and density, T-even bacteriophages move in centrifugal fields with sedimentation velocities unlike those of any other materials present in the host bacterium. Hence, phage particles can be readily separated from the general debris present in lysates by differential centrifugation (cf. Chapter 3, Vol. I). Second, because of the attachment organs resident in their tail, bacteriophages are specifically adsorbed to bacterial cells, so that if a suspension of sensitive bacteria is introduced into a phage lysate, only the phage particles but not the host-cell debris will be fixed (cf. Chapter 6, Vol. II). Finally, because bacteriophages are generally good antigens and exhibit a rigorous serological autonomy, they can be precipitated specifically with antiphage serum, so that if antiphage serum previously exhausted against bacterial host material is added to a phage lysate, the phage particles but not the debris will be precipitated by the antigen-antibody reaction (cf. Chapter 10, Vol. I). It is, therefore,

possible to inquire whether during the latent period, and in particular during the eclipse, there exist inside infected bacteria any incomplete particles which are not yet infective but which already possess one or more of these three attributes of the mature virus. Such investigations are greatly facilitated by the use of radioactive tracers, which make possible the search for such incomplete material through analysis of lysates for the presence of noninfective but sedimentable, adsorbable, or antiserum precipitable radioactivity.

Experiments have been carried out along these lines in which bacteria growing in media labeled either with radiophosphorus  $P^{32}$  or radiosulfur  $S^{35}$  were infected and lysed prematurely at various times after infection (Maaløe and Stent, 1952; Maaløe and Symonds, 1953).<sup>1</sup> The results with  $P^{32}$  were negative, in that during the eclipse no phosphorylated material can be found which is sedimented, adsorbed, or antiserum-precipitated like the parent phage. After the end of the eclipse, furthermore, the only phosphorylated structures found to be endowed with these properties are the infective progeny particles themselves. In other words, there do not appear to exist any incomplete, DNA-containing phage particles which possess either the size and shape, the attachment organs, or the antigenicity of the intact virus (Maaløe and Stent, 1952). The results with  $S^{35}$ , however, were positive, in that several minutes before the end of the eclipse, noninfective sulfurylated material specifically precipitable by antiphage serum was found to be present (Maaløe and Symonds, 1953). If the amount of precipitable phage antigen detected at various times after infection is expressed in multiples of the total amount of sulfur contained in a single mature bacteriophage, or *phage equivalents*, then approximately 10–20 phage equivalents of phage antigen already exist within the infected cell when the first mature progeny make their appearance. The amount of incomplete, or *surplus antigen*, present after the termination of the eclipse can be estimated by subtracting the number of infective phages found by infectivity assays from the total number of phage equivalents of antigenic sulfur recovered. The surplus antigen is then seen to attain a maximum of 30–40 phage equivalents per infected cell 20 minutes after infection and to remain at this level throughout the remainder of the latent period. This noninfective, antigenic sulfur resides in particles which are smaller, and hence less sedimentable, than the mature virus particles, but nevertheless sufficiently large to be sedimented by high-speed centrifugation

<sup>1</sup> As has already been discussed in detail in previous chapters, bacteriophages are composed of roughly equal proportions of deoxyribonucleic acid (DNA) and protein, of which the former harbors practically all of the phosphorus of the particle (in the polynucleotide phosphate diester bonds) and the latter all of the sulfur (in the amino acids methionine and cysteine). Hence, phages grown in a medium containing either  $P^{32}$  or  $S^{35}$  are labeled only in their DNA or only in their protein, respectively.

(Maaløe and Symonds, 1953; Hershey *et al.*, 1955; Watanabe, 1957). At least a fraction of these incomplete sulfurylated phage antigens must be a precursor of the mature phage progeny; not only are the antigens found to make their intracellular appearance prior to that of any infective particles, but it can also be shown by means of experiments in which the  $S^{35}$  label is either added to or withdrawn from the infected culture at various times of the latent period that some sulfur first present in incomplete antigens is later incorporated into intact viruses (Maaløe and Symonds, 1953; Hershey *et al.*, 1955).

## 2. Serum-Blocking Power

One may search for the presence of "incomplete" phage antigens, not only by virtue of their precipitability by antiphage serum, but also by virtue of their ability to *interfere* with the antiserum neutralization of a test population of the same phage strain. For any substance which has the same antigenic structure as the neutralizing antigen of the phage can combine with and absorb out the neutralizing antibodies of an antiphage serum, thus diminishing the neutralizing potency of the serum. The amount of such serum-blocking power, or SBP, can be estimated by comparing the reduction of the neutralizing titer produced by prior treatment of the serum with an unknown preparation with that produced by treatment with a known number of intact phage particles. One phage equivalent of SBP thus corresponds to the reduction in neutralizing activity produced by a single phage particle. Burnet (1933) discovered that after removing the infective particles from T-even lysates<sup>1</sup> by ultrafiltration, there is still SBP material present in the filtrate. These observations were confirmed by DeMars (1955) who found that from 10 to 25 % of the total SBP of a T-even lysate is in an "incomplete" form, i.e., does not form part of intact phages, being ultrafiltrable and nonsedimentable in centrifugal fields which would bring down phage particles. DeMars also examined the intracellular appearance of SBP in bacteria infected with T-even phages under one-step growth conditions and lysed artificially at various times after the onset of phage development. This experiment showed that the first, nonsedimentable SBP appears between 9 and 10 minutes after infection, or 2-3 minutes prior to the first infective progeny. At later times, the SBP activity was found to continue to increase along with the rise in progeny titer, although the total amount of SBP is always in excess of that attributable to intact phages. Since the neutralizing antigens reside in the tail of the T-even particle (Lanni and Lanni, 1953), it would seem that the "incomplete" SBP structures detected in these experiments represent parts of the phage tail protein. As such they are probably included in the antiserum precipitable surplus antigens described in the preceding section.

<sup>1</sup> Burnet's experiments actually concerned phage C16, which is closely related to the T-even group (Adams, 1952).



### 3. Morphology

It is also possible to search for incomplete phages by means of the electron optical observation of structures whose morphology bears some resemblance to the characteristic shape of mature bacterial virus particles. Wyckoff (1948) and Herčík (1950a,b,c) obtained electron micrographs of crude lysates of phage-infected bacteria in which, besides the typical tadpole-shaped mature bacteriophages, a variety of objects can be discerned whose form suggests they represent incomplete forms of the virus. Prominent among these are certain circular objects of about the same diameter as the electron-dense head of the mature phage particle, but differing from the latter by having no tail and by being either ring-shaped or "empty." That these rings, or "doughnuts," are indeed related to intracellular phage growth could be demonstrated by Levinthal and Fisher (1952), who broke open phage-infected bacteria by explosive decompression at various times after infection and counted the number of various objects visible on their electron micrographs. Levinthal and Fisher found that at early stages of the latent period no structures are apparent inside the infected host cell which cannot also be seen in uninfected control bacteria similarly disrupted by decompression. About 3 minutes before the emergence of morphologically intact phages and the termination of the eclipse, however, the doughnuts make their appearance and then increase in number at about the same rate as the complete phage particles which follow them, until a maximum of about 35 doughnuts per infected bacterium has been attained. The number of doughnuts then remains at this level, while the number of intact phage particles per cell rises to the neighborhood of 100. Anderson *et al.* (1953) subsequently found that the doughnuts are, in fact, the *empty heads* of phage particles, which look like flat discs rather than rings when the specimens for electron microscopy are dried by methods which avoid surface tension distortions. The doughnuts are not adsorbed to sensitive bacteria, in agreement with the fact that the empty heads have no tail, the normal attachment organ of the phage. They also do not possess any neutralizing antigens and are, therefore, not identical with the incomplete serum-blocking antigens described in the previous section. They do fix complement in the presence of antiphage serum, however, and contain, therefore, part of the antigenic structures of the phage. The mass of the doughnuts is of the order of 25 % of that of the whole virus and comprises most of the protein of the virus (i.e., about 75 % of the total sulfur) and very little of the nucleic acid (i.e., less than 15 % of the total phosphorus) (DeMars *et al.*, 1953). Further experiments by Kellenberger and Séchaud (1957) confirmed Levinthal and Fisher's kinetic studies on the appearance of empty phage heads and also demonstrated the intracellular presence of certain "rods." These rods resemble the phage tail in structure and dimension and, unlike the doughnuts, can be fixed to the phage receptor sites of bacterial cell





FIG. 1. Micrograph of a crude T2 lysate, showing morphologically intact phages, empty heads, rods, and, in the upper part of the micrograph, a group of filaments which may be DNA. (Reproduced by permission from Kellenberger and Séchaud, 1956.)



walls. Kellenberger and Kellenberger (1957), finally, made electron optical observations on the production of phage-related structures in single infected bacteria and found that previous conclusions concerning the appearance of doughnuts drawn from study of mass lysates are also valid at the level of the individual cell. Figure 1 shows an electron micrograph in which morphologically intact phages, empty heads, and rods are visible.

In contrast to the serum-precipitable, sulfurylated, incomplete phage antigens, the precursor nature of the SBP antigens and of the empty heads has not yet been clearly established. To be sure, both of these incomplete materials are found within the phage-infected cell prior to the appearance of any infective progeny particles, but it is difficult to demonstrate that incomplete SBP antigens or empty heads present at an early stage of the latent period form part of intact viruses later on. The fact that the intracellular concentration of both of these incomplete materials reaches a maximum soon after the termination of the eclipse has been interpreted in favor of the precursor view by supposing that this constant level reflects a steady state of depletion by conversion into mature progeny and replenishment by renewed synthesis (Levinthal and Fisher, 1952; DeMars, 1955).

#### 4. *Phage-Specific Nucleic Acid*

Although the experiments discussed so far demonstrate the appearance of noninfective proteinaceous structures already during the eclipse, they fail to reveal any incomplete forms of the phage DNA, the other major component of the mature virus. Fortunately, in the case of the T-even bacteriophages, it is possible to examine infected bacteria for any phage-specific nucleic acid by taking advantage of the discovery that the DNA of these phage strains contains the unusual pyrimidine, 5-hydroxymethylcytosine (HMC), in place of the more common base, cytosine, of the DNA of the *E. coli* host bacterium (Wyatt and Cohen, 1952). Hershey *et al.* (1953), therefore, infected *E. coli* bacteria under the conditions of the one-step growth experiment, extracted the DNA of the infected complexes at various times after the onset of phage development and analyzed the extract for its content of HMC, cytosine, and the other purine and pyrimidine bases. These analyses then permitted an estimation of the number of phage equivalents of HMC-containing DNA present at any time, based on the total nucleic acid and relative HMC content of the mature phage particle. Vidaver and Kozloff (1957) later repeated the study of Hershey *et al.*, paying, however, closer attention to the very earliest stages of the latent period in order to establish more precisely the onset of synthesis of HMC-containing DNA. The results of these experiments can be summarized by stating that the synthesis of bacteriophage DNA already commences between 6 or 7 minutes after infection and then proceeds so rapidly that a few minutes later at the termination of the eclipse, when the



first infective progeny make their intracellular appearance, already 40 to 80 phage equivalents of HMC-containing DNA are present within the infected cells. The synthesis of this DNA continues throughout the remainder of the latent period, there being always an excess of the number of phage equivalents of phage DNA over the total number of mature progeny viruses which accumulate during this time. The excess phage DNA which does not form part of intact progeny particles is "free" in the sense that after artificial lysis of the infected cell, it is sensitive to the action of deoxyribonuclease and does not sediment in centrifugal fields capable of sedimenting mature phages (Hershey, 1953a).

It is thus apparent that the formation of both the protein and the nucleic acid of the descendants of the infecting bacteriophage commences during the eclipse. Protein and nucleic acid moieties, however, first appear in separate forms within the infected cell prior to a process of *maturation* by which these two principal components of the virus are united to constitute the infective unit.

### *B. The Precursor Pool*

In order to account for his discovery of multiplicity reactivation after UV irradiation, Luria (1947) proposed that the genome of the T-even phages is comprised of a number of subunits, which multiply independently of one another and generate an intrabacterial *subunit pool* from which a full genetic complement is subsequently withdrawn for each progeny particle. Some years later, Visconti and Delbrück (1953) were similarly led to propose, on the basis of their study of the mechanism of genetic exchange of bacteriophages, that, prior to their maturation into infective progeny, the genetic structures of the vegetative phage find themselves in a *mating pool*, in which they undergo recombination and from which they are withdrawn at random for maturation into resting, infective progeny (cf. Chapter 8). Contemporaneously with these genetic investigations, isotopic tracer studies of the kinetics of synthesis and assembly of the viral components had commenced. These studies, as we shall see now, likewise led to the idea that the incomplete viral components, phage DNA and phage protein, exist in pools prior to their being put together into mature phage particles at the termination of the eclipse.

#### *1. Origin of Progeny Nucleic Acid*

Some theories of the nature of phage multiplication envisioned that there are already present within the normal host bacterium bacteriophage precursors, whose metamorphosis into mature bacteriophages is merely triggered off by the infecting phage particle (Krueger and Scribner, 1939). This view was dispelled by an experiment of Cohen (1948b), which has since

served also as a model for resolving other precursor questions e.g., that which had posed itself to the students of induced enzyme synthesis (Hogness *et al.*, 1955). Cohen grew two bacterial cultures, one in a medium labeled with a given specific activity of  $P^{32}$  and the other in a nonlabeled medium. After growth, the bacteria were centrifuged out of their original culture fluids, the labeled cells resuspended in nonlabeled growth medium, and the nonlabeled cells resuspended in labeled growth medium. Both cultures were then multiply infected with T-even phages, incubated until complete lysis, and the phage yields isolated, purified, and assayed for their specific  $P^{32}$  contents. The result of Cohen's experiment was that the DNA-phosphorus of the phages grown on labeled bacteria in nonlabeled medium possessed only 1/3 of the specific activity of the  $P^{32}$  introduced, whereas the DNA-phosphorus of the phages grown on nonlabeled bacteria in labeled medium possessed 2/3 of the specific activity of the  $P^{32}$  introduced. Cohen thus demonstrated that most of the phage DNA is synthesized from materials still in the growth medium at the moment of infection, i.e., that the phage particles cannot have been derived from pre-existing bacterial precursors. Cohen's experiment was later repeated, using the nitrogen isotope  $N^{15}$  as the label, by which means it could be shown that most of the nitrogen of the phage DNA, like its phosphorus, is also derived from materials still in the growth medium at the moment of infection (Kozloff *et al.*, 1951). Contrary to what had been supposed at first (Cohen, 1948b), the fraction of the atoms of the phage DNA assimilated before infection, or the *bacterial contribution*, is not preferentially derived from low molecular weight intermediates of bacterial nucleic acid metabolism present in the host cell at the moment of infection (Kozloff and Putnam, 1950; Stent and Maaløe, 1953). Instead, it could be shown by infecting bacteria labeled with radiocarbon  $C^{14}$  only in their pyrimidines (Weed and Cohen, 1951), or exclusively in their thymine (Kozloff, 1953; Hershey *et al.*, 1954) that the bacterial nucleic acids, or, more specifically, the bacterial DNA, is the chief provenance of the bacterial contribution. (A minor fraction of the bacterial contribution is also derived from the host ribonucleic acid (Hershey *et al.*, 1954).) The utilization of host nucleic acid for the bacterial contribution involves breakdown of the bacterial DNA into low molecular weight substances subsequently repolymerized into phage-specific polynucleotides (Hershey *et al.*, 1953, 1954). Since the composition of the bacterial DNA is very different from that of the T-even phages, in particular in that the former contains cytosine and the latter HMC, any biochemical route for the bacterial contribution other than breakdown and resynthesis would, in any case, be difficult to imagine. In the course of the latent period more than 85 % of the host (i.e., cytosine-containing) DNA disappears, most of which ultimately reappears in phage (i.e., HMC-containing) DNA (Hershey, 1953a; Hershey *et al.*, 1954), host cytosine having been converted into viral HMC (Cohen, 1953).



The inferred extensive breakdown of the host DNA is in harmony with direct cytological observations on T-even infected bacteria, which reveal that the host nucleus disappears at early stages of intracellular phage development (Luria and Human, 1950; Murray *et al.*, 1950).

The kinetics of assimilation of the phosphorus of phage DNA have been studied by another extension of Cohen's original tracer experiment. For this purpose,  $P^{32}$  label was either added to a nonradioactive bacterial culture or withdrawn from a  $P^{32}$ -labeled bacterial culture at various times before or after its infection under one-step growth conditions. The fraction of the total phage phosphorus assimilated by the time of addition or removal of the  $P^{32}$  label could then be estimated from the specific  $P^{32}$  activity of the DNA-phosphorus of the phage yield produced (Stent and Maaløe, 1953). The result of this experiment was that, prior to infection, the bacteriophage phosphorus is assimilated at the rate of bacterial growth, indicating once more that end products of bacterial synthesis, like the nucleic acids, and not metabolic intermediates, are the source of the bacterial contribution. Immediately after infection, however, the rate of phage phosphorus assimilation was seen to increase sharply, indicative of a sudden alteration in the metabolism of the infected cell which channels almost all of the newly assimilated phosphorus atoms into the pathway of phage DNA synthesis. Assimilation was found to be complete within 20 minutes after infection, although mature progeny phage continue to make their appearance for at least 10 more minutes. A further variable was introduced into this experiment by inducing lysis of the infected cultures from time to time and determining how the phosphorus assimilated at different stages of the growth cycle is distributed over phage progeny maturing at various times. The results obtained indicated that the bacterial contribution appears preferentially, though not exclusively, in the first progeny to mature, that phosphorus atoms assimilated after infection spend an average "development time" of 14 minutes in the cell prior to their incorporation into the DNA of infective phages, and that individual development times of different phosphorus atoms are widely distributed around this average. This apparent mixing of phosphorus of the bacterial contribution and of phosphorus assimilated at various times after infection led to the idea that phosphorus of the phage DNA passes through one or more intrabacterial precursor *pools*, whose size and number is reflected by the observed degree of mixing (Stent and Maaløe, 1953). Hershey (1953a) carried out further experiments on the kinetics of the transport of phosphorus from the culture medium and the bacterial DNA to the viral progeny. He inferred from his observations that at the end of the eclipse period the DNA of the phage particles about to mature is withdrawn from a pool amounting to 50–100 phage equivalents of phage precursor DNA, which pool then maintains a more or less constant size during the remainder of the latent period, while DNA synthesis and

maturation keep pace with one another. The kinetic pool size estimate is thus in excellent agreement with the value of 40–80 phage equivalents of HMC-containing DNA obtained by direct chemical analysis, which we had already noted as being present within the infected cells. Hershey could show, furthermore, that maturation is *efficient*, in that about 90 % of the labeled phosphorus introduced early into the pool is later incorporated into intact phages, and that maturation is *irreversible*, in that phosphorus in mature, intracellular phages does not undergo exchange with the phosphorus of the DNA precursor pool.

## 2. *Origin of Progeny Protein*

The synthesis of the phage protein was studied by means of yet another modification of Cohen's tracer experiment. For this purpose,  $N^{15}$  or  $C^{14}$ -lysine-labeled bacteria were infected with T-even phages and phage development allowed to proceed in nonlabeled medium before the protein of the phage progeny was analyzed for its specific content of the labeled isotope. It was found that very little, if any, of the viral protein is derived from the protein of the host cell, i.e., that the bacterial contribution to the protein is much less than to the DNA of the bacteriophage (Kozloff *et al.*, 1951; Siddiqi *et al.*, 1952). The kinetics of assimilation from growth medium of the raw materials for the phage protein were studied by Hershey *et al.* (1954), who exposed infected bacteria to  $S^{35}$  label during 5-minute intervals at various stages after the onset of phage development and then determined the amount of  $S^{35}$  subsequently incorporated into virus and into the general intrabacterial protein. This study showed that the total amount of label which enters intrabacterial protein during the 5-minute exposure to labeled growth medium is more or less the same at all stages of the latent period, as well as also being equal to the labeling attained during the same interval by an uninfected bacterial culture, thus confirming an earlier finding by Cohen (1948a) that total protein synthesis proceeds in the host cell at the same rate before as after infection. The proportion of the total  $S^{35}$  assimilated which finds incorporation into virus protein, however, varies greatly at different stages of the latent period. Very little of the sulfur assimilated during the first 5 minutes after infection ultimately enters the protein of the phage progeny, whereas 50–60 % of the total sulfur assimilated during any 5-minute interval after the tenth minute of phage development is directed toward mature virus particles. These results indicate that two classes of protein, one a phage precursor and the other not, are synthesized in the infected host cell and that the maximal rate of precursor protein synthesis is not reached until the latter part of the eclipse period. It can be inferred from considerations similar to these already described for the interpretation of the  $P^{32}$  assimilation data, that sulfur atoms spend an average development time of about 2 minutes

between assimilation from the medium and incorporation into antigenic phage precursor protein, and a further development time of 8 minutes until their incorporation into mature virus particles (Maaløe and Symonds, 1953; Hershey *et al.*, 1954, 1955). Thus, there also exists a *pool* of phage precursor protein from which material begins to be withdrawn after the termination of the eclipse for the formation of mature virus particles. The size of this precursor protein pool appears to amount to about 15 phage protein equivalents at the termination of the eclipse (Hershey and Melechen, 1957), although it will be recalled that about two to three times as many incomplete phage antigens and electron optically visible doughnuts have been observed to be present in each cell at that time. This apparent discrepancy in intracellular amount of different types of protein precursors could be either due to possible variations in physiological conditions of growth employed by the different investigators whose work is being compared here, or due to the fact that some of the incomplete proteinaceous materials, in particular the SBP structures and the doughnuts, are not really precursors of the mature progeny particles after all. In any case, the pool of phage precursor protein appears to be smaller than the pool of phage precursor nucleic acid; this difference in pool sizes can be exaggerated further by chloramphenicol treatment, which, as shall be mentioned soon, permits the synthesis of phage precursor nucleic acid in the absence of *any* phage precursor protein. Hence, the mechanism of synthesis of the phage precursor nucleic acid cannot be such that the phage DNA is necessarily formed inside particles possessing a phage precursor protein membrane (Hershey and Melechen, 1957).

### C. Maturation

The enclosure of the phage precursor nucleic acid into a precursor protein head membrane and endowment with a precursor protein phage tail thus appears to represent one of the last acts of intracellular virus growth, i.e., the *maturation* of the infective unit. "Even when forced into this possibly oversimplified scheme," wrote Hershey (1957a), "the maturation process presents a more complicated problem, conceptually at least, than vegetative reproduction. In this respect it resembles, of course, morphogenetic problems in general. So far, this aspect of phage growth has been attacked only as a side issue to what have seemed to be more promising opportunities."

#### 1. Phenotypic Mixing

A series of genetic experiments relevant to the maturation process had their origin in an observation by Delbrück and Bailey (1946) of anomalous host range behavior of phages liberated by bacteria mixedly infected with T4 and T2. Novick and Szilard (1951) investigated this anomaly further and found that among the progeny of T4  $\times$  T2 crosses there exists particles which

are *phenotypically* like T4, inasmuch as they have the T4 host range, but are *genotypically* like T2, inasmuch as their progeny have the T2 host range. The process by which phage particles arise possessing the host range genome of one but the host range phenotype of another parent is called phenotypic mixing (Hershey *et al.*, 1951b). Phenotypic mixing has been observed not only for the host range character but also for neutralizing antigens and adsorption-cofactor requirements (Streisinger, 1956; Brenner, 1957). From detailed studies of the mechanism of phenotypic mixing, Streisinger (1956) concluded that among the issue of a mixed infection, host range and neutralizing antigen phenotypes are randomly associated with the corresponding genotypes. Since the proteinaceous phage tail is the site of adsorption to the sensitive host (Anderson, 1953; Kozloff and Henderson, 1955; Kellenberger and Arber, 1955) and antiserum neutralization (Lanni and Lanni, 1953), it is evident that the phenotypes involved in phenotypic mixing represent certain structural features of the phage protein. The genotype of these traits, on the other hand, probably resides in the phage DNA molecules, which, as shall soon be considered, bid fair as the germinal substance of the extracellular virus particle. One may envision, therefore, that the union of the viral DNA with the viral (tail) protein proceeds by random association of materials from the phage DNA and phage protein precursor pools.

## 2. Proflavine

One other line of experimentation relevant to the maturation process has shown that it is possible to interfere with maturation by means of drugs. Foster (1948) observed that in the presence of the acridine dye, *proflavine*, phage-infected bacteria lyse after the normal latent period but release no infective progeny. If proflavine is removed from the growth medium a few minutes before the onset of lysis, however, a considerable burst of infective progeny is released, suggesting that proflavine blocks a rather late step of phage reproduction. Electron optical, immunological, and chemical studies support this view, since noninfective proflavine lysates do contain the "incomplete" phage constituents mentioned in an earlier section. Thus, it has been found that, in the presence of proflavine, empty phage heads or doughnuts, complement-fixing (CF), serum-blocking (SBP) phage antigens, and phage (i.e., HMC-containing) DNA make their intracellular appearance in amounts and with kinetics not unlike those characteristic of infected bacteria growing in the absence of the drug. No structures are present in proflavine lysates, however, which exhibit the morphology of intact phage particles (DeMars *et al.*, 1953; DeMars, 1955; Kellenberger and Kellenberger, 1957). One would thus like to believe that while proflavine allows the synthesis of all known major constituents of the phage, it inhibits in some way their assembly into the mature virus. The observation of Kellenberger and Séchaud



(1957) that the number of incomplete phage protein doughnuts per cell is *no greater* in proflavine than in normal lysates seems puzzling from this point of view, since one would have expected an intracellular accumulation of these incomplete structures if only their incorporation into mature phage but not their synthesis had been arrested by the drug.

#### IV. SYNTHESIS OF NONPRECURSOR MATERIALS

##### *A. Ribonucleic Acid*

Thus far in our discussion of phage multiplication we have been concerned with the synthesis inside the infected bacterium of bacteriophage "precursors," i.e., of structures which upon their maturation will constitute the matter of the progeny phages. We shall now turn our attention to some "nonprecursor" materials connected with the reproductive processes, i.e., substances which, though synthesized by the phage-infected bacterium, are not ultimately incorporated into infective virus particles.

Ribonucleic acid (RNA) is the principal phosphorylated constituent of *E. coli* and hence the destination of most of the phosphorus assimilated by the cells during their normal growth. The rapid RNA synthesis of the bacteria comes to a sudden halt upon their infection by T-even phages and very little increase in total RNA content of the infected culture can be discerned during the remainder of the latent period (Cohen, 1947; 1948a; Manson, 1953). Tracer experiments in which  $P^{32}$  is added to the bacterial culture only after infection suggested, however, that an appreciable amount of the label does enter an RNA fraction during phage growth, i.e., that some RNA synthesis might be occurring in the infected cells after all and that the previously observed post-infection constancy in RNA content represents a balance of breakdown and resynthesis (Hershey, 1953a). More complete studies, in which was examined the incorporation of  $P^{32}$ , not only into RNA, but also into the four individual ribonucleotides—cytidylic, adenylic, uridylic, and guanylic acids—definitely established the existence of an RNA synthesis during intracellular phage growth (Volkin and Astrachan, 1956a, 1957). These studies showed, not only that radioactive ribonucleotides can be isolated from the digest of the RNA of the infected cells, but also that the radioactive label does not enter each of the four ribonucleotides at the same relative rate at which the ribonucleotides of uninfected *E. coli* bacteria would have acquired  $P^{32}$  under analogous labeling conditions, i.e., that the postinfection synthesis concerns a species of RNA of base composition different from that of the over-all host cell RNA. The amount of postinfection RNA present at any time is rather small compared to the total RNA content of the bacterium but, if expressed in multiples of the weight of nucleic acid per T-even phage particle, nevertheless attains a level

of about 20 phage equivalents per cell. It could be shown, furthermore, in experiments in which the  $P^{32}$  label was either added to or withdrawn from the culture at various times after infection, that phosphorus atoms continuously enter and leave this postinfection RNA species, i.e., that this fraction is in a state of rapid metabolic turnover, in contrast to the RNA of normal, rapidly growing *E. coli* cells for which no such turnover exists (Hershey, 1954). Whatever may be the importance of this postinfection RNA for phage multiplication, it cannot be a direct precursor of the infective progeny, since the extracellular T-even particles contain no RNA (Putnam, 1953; Volkin and Astrachan, 1956b).

### B. "Early" Protein

We have already noted that, in contrast to the immediate cessation of the net synthesis of RNA, the formation of bacterial protein continues unimpaired after infection of the host cell. Since the synthesis of *phage precursor protein*, however, does not commence until the latter part of the eclipse period, it might be thought that the protein synthesized within the infected cell in the time intervening between infection and the onset of precursor manufacture is not directly connected with the process of phage reproduction. The experiments to be recounted now demonstrate that, on the contrary, a protein species arises among these nonprecursor proteins formed immediately after infection, which is an essential factor of intracellular phage development. Cohen and Fowler (1947) found that there is no phage growth if the amino acid analog, 5-methyl-tryptophan, which arrests protein synthesis by blocking the biosynthesis of tryptophan (Beerstecher, 1954), is added to bacteria at the moment of their infection with T-even phages. From this work it already appeared that the observed interference with phage growth could not be due just to the inhibition of synthesis of the phage protein, but must represent an interference with a very early step in the reproduction process, since net synthesis of DNA by the infected cell also is suppressed. After removal of 5-methyl-tryptophan from the infected culture, phage development commences, leading to a burst of progeny phages after a normal latent period reckoned from the time of removal of the inhibitor. Subsequent refinements and extensions of these studies showed that there is no synthesis of phage DNA or onset of phage development in infected bacteria in which protein synthesis has been suppressed from the very start either by the presence of an inhibitory amino acid analog, by the absence of required amino acids, or by the presence of the antibiotic, chloramphenicol. If, however, the bacteria are infected under normal conditions and protein synthesis is stopped by any of these methods only a few minutes after the start of intracellular phage development, then the synthesis of HMC-containing phage DNA does proceed in the absence of any further formation of protein, the *rate* of such DNA synthesis

being the greater the later protein synthesis has been arrested. Nevertheless no infective progeny particles appear under these conditions until the synthesis of phage precursor protein is allowed to take place by once more removing the inhibitory conditions (Burton, 1955; Melechen, 1955; Tomizawa and Sunakawa, 1956; Hershey and Melechen, 1957). It seems, therefore, that the formation of a *nonprecursor* protein is required before replication of the bacteriophage DNA can commence. Once some of this protein has been made, DNA synthesis can proceed in the absence of further protein synthesis. One might suppose that this protein is an enzyme required for the synthesis of some precursor of the phage DNA, e.g., for HMC, were it not for the observation of Tomizawa and Sunakawa (1956) that the ultraviolet light sensitivity of the T-even infected bacteria does not decrease further after the addition of chloramphenicol, even though more and more phage DNA accumulates within the cell.<sup>1</sup> Rather, if the DNA manufactured under these conditions is "good" phage DNA, then it would seem likely that the early, nonprecursor protein plays a more profound role in the reproductive process of the vegetative phage than merely being an enzyme of the intermediary metabolism of the infected cell. The nature of the phage DNA synthesized in T-even infected bacteria to which chloramphenicol has been added a few minutes after infection has been investigated by Hershey and Melechen (1957). By adding radiophosphorus P<sup>32</sup> to the infected cells only during the time of chloramphenicol inhibition, it could be shown that the DNA synthesized under these conditions does enter the progeny phages which appear after protein synthesis has been once more allowed to proceed. Furthermore, the "normal" DNA formed after removal of the chloramphenicol appears to be diluted in the phage precursor nucleic acid pool constituted by the previously synthesized "chloramphenicol-DNA" before it in its turn finds incorporation into mature progeny particles. The DNA formed in the absence of protein synthesis thus appears to be "good" in the sense of being a genuine phage precursor.

Not only is it possible for phage DNA to be synthesized in the absence of protein synthesis, but the converse is also true: phage protein can be synthesized in the absence of concomitant phage DNA synthesis. This was established by an experiment in which infected bacteria were irradiated with ultraviolet light (UV) at various stages of the latent period, S<sup>35</sup> label added to the growth medium immediately after irradiation, and the total amount of radioactivity precipitable by antiphage serum, i.e., intracellular antigenic phage protein, estimated after further incubation of the infected culture (Watanabe, 1957). It was observed in this experiment that no phage antigen is ever formed in

<sup>1</sup> This statement refers to the experiment of Luria and Latarjet (1947), in which the progressive reduction in UV sensitivity of the infective center constitutes an index of the progress of intracellular phage development (cf. Chapter 10, Vol. II).

bacteria infected with phages already UV-inactivated prior to infection or in infected bacteria whose survival as infective centers has been destroyed by UV-irradiation at early stages of the eclipse period. If the infected bacteria are UV-irradiated at later stages of the eclipse period, however, when some antigenic precursor protein is already present in the cells, then phage antigen *continues* to be synthesized at a rapid rate even though further DNA synthesis and, *a fortiori*, production of infective progeny has been abolished by the irradiation. Thus it seems that while at the outset of the intracellular phage growth DNA and protein syntheses are connected with one another in some necessary way, so that stopping one will stop both, the two processes become independent of each other once the mechanism for formation of phage-specific protein has been established within the infected cell.

## V. FATE OF THE INFECTING PHAGE PARTICLE

### *A. Functional Differentiation and Injection*

The work discussed in the preceding sections has given some insight into the manufacture of the substance of the progeny virus particles within the phage-infected cell. In this discussion it became clear that the two principal components of the progeny phages, i.e., protein and DNA, are synthesized separately and that their *union* is the all but last act of the intracellular reproduction process. From the investigations to be recounted now, which are concerned with what actually happens to the parental phage particle when it infects the host cell and presides over the reactions leading to its manifold reduplication, it has been learned that the *separation* of protein and DNA of the infecting virus is, in fact, also the first act of the intracellular reproduction process. These experiments can be thought to have taken their inception with the observation that T-even bacteriophage particles are inactivated by osmotic shock (Anderson, 1949, 1953). Osmotic shock engenders the release of the DNA from the proteinaceous membrane of the phage head, leaving behind DNA-free "ghosts" still capable of adsorbing to and even killing bacterial host cells (Herriott, 1951). The osmotic release from the phage head makes the DNA accessible to such degradative enzymes as deoxyribonuclease (DNAase), from whose action the head membranes had previously protected it. Contemporaneously with the finding of osmotic disruption, the phenomenon of "superinfection breakdown"—to be discussed in more detail below—had been discovered; it indicated that, as in osmotic shock, so also in the process of infection the DNA of the parental phage particle becomes exposed to the action of degradative enzymes (Lesley *et al.*, 1950, 1951). Hershey and Chase (1952) then demonstrated in the wake of these observations the existence of independent functions of the two viral moieties, protein



and DNA, by means of their "Blendor experiment." This experiment consists of infecting bacteria with  $P^{32}$ -,  $S^{35}$ -, or  $C^{14}$ -labeled phage particles, separating the infected cells from any unadsorbed phages by centrifugation and resuspension in a salt-poor medium, and agitating the infected suspension violently for a short time in a Waring Blendor. The infected bacteria are then centrifuged once more in order to examine how much of the adsorbed radioactivity has been stripped off the cells by agitation, i.e., how much remains in the supernatant fluid, and how much has still remained attached to the cells, i.e., is sedimented into the bacterial pellet. The result of this experiment was that more than 80 % of the viral sulfur (mostly in methionine) and amino acid carbon is removed by the Blendor treatment, but that more than 80 % of the viral phosphorus or purine-pyrimidine carbon remains attached to the cells. Viability assays are also made before and after agitation; they show that, in spite of the extensive removal of viral protein, practically all of the infected bacteria can continue to produce mature phage progeny. It appears from these results that most of the viral protein remains at the surface of the infected cell and that most of the viral DNA enters the cell at the outset of the infection. Hershey and Chase (1952) further substantiated the inference that in the process of entering the host cell the phage DNA leaves its protein envelope by showing that adsorption of phages to bacterial cell-wall debris or to heat-killed bacteria, or alternately freezing and thawing phage-infected cells sensitizes the DNA of the infecting virus particles to the action of DNAase. (In the case of adsorption to bacterial cell-wall debris, the phage DNA is liberated into the medium; in the case of heat killed or frozen-thawed bacteria, the phage DNA enters a host cell whose membrane has been rendered permeable to the DNAase.) Hence, the bulk of the phage protein appears to be relieved of any further function in the intracellular reproduction processes after the proteinaceous tail has made possible the specific adsorption of the virus particle to the bacterial surface and the DNA has been safely "injected" into the interior of the host cell (cf. Chapter 6).

However, before dismissing the phage protein from further consideration altogether, some mention must be made of that 20 % of the amino acid radioactivity which is not removed from the infected cells in the blendor experiment. Much of this protein fraction probably represents these parts of the phage tails which adhere too firmly to the bacterial cell wall to be shaken loose, for electron microscopic observations show that the materials stripped from the infected cells by blendor treatment are phage ghosts, which are intact except for shortened tails (Levinthal and Fisher, 1953). An indication that, at most, a small portion of this nonremovable 20 % of the phage protein actually enters the infected cell is that less than 1 % of the parental methionine or lysine reappears in the protein of the progeny phage particles (Hershey and Chase, 1952; French, 1954)—in contrast to the atoms of the

phage DNA, which *do* enter the cell and which, as will be seen below, *are* transferred efficiently to the DNA of the progeny phages. In fact, Hershey (1955) has been able to fix an upper limit of 3 % for the amount of total phage protein which could enter the infected cell and play some role in the course of intracellular phage reproduction. An "acid-soluble" peptide has, however, been identified, which *is* injected into the host cell by the parental phage but not transferred to the progeny. This peptide amounts to about 2 % of the total phage protein and contains no sulfur, being composed mainly of lysine, glutamic acid, and aspartic acid. The phage also injects some polyamines which are transferred as such to the progeny particles (Hershey, 1957b; Ames *et al.* 1958).

The discovery that the DNA, and not the protein, of the infecting phage enters the host cell at the moment of infection thus clearly shows that it must be the DNA which is the carrier of the genetic continuity of the virus particle, i.e., the germinal substance of the extracellular, resting phage. (The 20 % of the phage DNA found to be strippable in the blender experiment probably represents a minor fraction of the phage population, which does not infect the host properly and which fails to inject its nucleic acid core (Hershey, 1953b).) Such an assignment of a genetic role to the viral DNA is in perfect harmony with notions derived from bacterial transformation experiments, in which the hereditary factors of a donor bacillus are transferred to the genome of a receiver cell through the exclusive vehicle of DNA molecules (Avery *et al.*, 1944). The release of the phage DNA from its protein envelope at the very moment of infection, furthermore, now readily accounts for the existence of an eclipse period at the early stages of intracellular virus development, when no infective particles whatsoever can be recovered by induced lysis of the host cell. For having just been divested of its attachment and injection organs, the DNA of the infecting phage is naturally unable to gain entrance into any further bacterial cells to which it may be presented in the infectivity test. It is actually not excluded *a priori* that phage DNA, like bacterial DNA in transformation experiments, *can* gain entrance into and infect bacterial cells directly. Experiments to test this possibility have generally led to negative results (Cohen, 1947), although it has been claimed that phage DNA can infect bacteria whose cell wall has been weakened by various treatments (Spizizen, 1957; Fraser *et al.*, 1957).

### *B. Transfer of the Parental Nucleic Acid*

#### *1. Extent and Specificity of Transfer*

In 1950, Putnam and Kozloff invented an experiment directed toward the question of how much, if any, of the substance of the DNA introduced into the host cell by the infecting bacteriophage reappears among the DNA of its

progeny. In this "transfer experiment," bacteria are infected under conditions of one-step growth with phage particles whose DNA is labeled with  $P^{32}$  or  $C^{14}$  and the phage yield issuing from these infected cells is harvested, purified, and assayed for its content of the parental radioisotopes. The outcome of Putnam and Kozloff's transfer experiment was that about 30–40 % of the radioactive atoms of the parental DNA are transferred to the progeny. Subsequent work employing improved experimental techniques confirmed the transfer of an appreciable fraction of the infecting DNA and revealed that the transfer efficiency can amount to as much as 50 %. The other, nontransferred 50 % of the labeled, parental DNA atoms of the lysate are usually found to be either attached to bacterial debris (10 %), or liberated into the growth medium in the form of macromolecular free DNA (30 %), or in low molecular weight, acid-soluble compounds (10 %) (Maaløe and Watson, 1951; French *et al.*, 1952; Hershey and Chase, 1952; Hershey, 1953a; Kozloff, 1953; Watson and Maaløe, 1953; French, 1954; Hershey and Burgi, 1956). In view of the inference that it is the DNA of the extracellular virus particle which carries its genetic continuity into the host cell, it does not seem farfetched to imagine that an understanding of the mechanism by which this transfer of DNA atoms from parent to offspring takes place might afford valuable insights into the nature of the reproductive processes. One may enquire, first of all, why the transfer is incomplete. Why do only 50 % of the parental DNA atoms reappear in the progeny? It is possible that phage reproduction involves no direct transfer of specific parental DNA at all, but that the parental DNA is degraded in the course of the latent period, the fragments introduced into the synthetic pathways of the infected cell and built up, along with other nucleic acid precursors, into new bacteriophage-specific progeny DNA. That this type of transfer *can* occur is demonstrated in experiments in which bacteria are mixedly infected with unlabeled T4 and  $P^{32}$ -labeled T3 phage particles. In spite of the fact that phage T3 is completely excluded, and its DNA presumably degraded under these conditions, some of its  $P^{32}$  label reappears among the T4 progeny (Kozloff, 1952, 1953; Watson and Maaløe, 1953). However, a closer study of the chemistry of transfer makes the hypothesis of transfer by breakdown and resynthesis less likely. For it has been found, not only that the phosphorus and all four pyrimidine residues of the parental DNA are transferred with equal efficiency, but also that addition of unlabeled thymidine and uridine to the phage-infected bacteria during intracellular phage growth does not suppress the transfer of parental, labeled pyrimidines to the progeny (in contrast to the incorporation into phages of pyrimidines derived from the bacterial host DNA, which incorporation is suppressed by similar addition of pyrimidine nucleosides to the infected culture) (Hershey *et al.*, 1954; Hershey and Burgi, 1956). The findings that the DNA from UV-irradiated parental phages carries its lesions with it in mixed infection with

nonirradiated phages and is to a large part transferred into *dead* progeny particles and that, in transfer experiments involving mixed infection of bacteria with genetically different parents, a considerable part of the parental label stays "true" to the genetic marker with which it was introduced are further powerful arguments against the notion of transfer by breakdown and resynthesis (Hershey *et al.*, 1954; Hershey and Burgi, 1956).

Another conceivable interpretation of the incomplete transfer might be that the phage DNA consists of two portions, one of which is transferred intact, while the other is not transferred at all. This idea was tested both by Maaløe and Watson (1951) and Hershey *et al.* (1951b), who carried the transfer experiment through two successive cycles of phage growth. They reasoned that if the transferred atoms represented a special "transferable" part of the viral DNA, then the labeled atoms introduced by the original parents should be concentrated in the transferable sector of the first generation progeny and hence be transferred to the second generation progeny with much higher efficiency during a second-growth cycle. In fact, the same transfer efficiency was found in both cycles. This experiment was later extended to a third generation transfer (Stent *et al.*, 1958) with results which can be schematized as follows:

Parent (label = 100 %) → 1st progeny (label = 50 %) →

2nd progeny (label = 25 %) → 3rd generation progeny (label = 12%)

One may conclude, therefore, that the DNA that is transferred neither originates from a preferentially transferable part which remains intact nor winds up preferentially in a part which is preferentially lost in the next infection. It would appear, rather, that the reasons for the incompleteness of the transfer must be sought among random losses experienced by the entire parental DNA in the course of infection, replication, and maturation, as well as among certain technical difficulties inherent in the transfer measurements themselves (Hershey, 1956; Hershey and Burgi, 1956). Further insight into the transfer processes can be obtained by breaking open bacteria infected P<sup>32</sup>-labeled phages at various stages of the latent period and examining the intracellular state of aggregation of the parental DNA. Such experiments show that during the eclipse most of the parental phosphorus remains part of free DNA fibers, the remainder being broken down to low molecular weight substances (Watanabe *et al.*, 1954). After the termination of the eclipse, the parental DNA is reincorporated into the first mature progeny phages making their intracellular appearance, each phage particle receiving on the average 2 % of the parental atoms. Particles maturing at later times receive less and less of the parental atoms, until the transfer is essentially complete by the time that the average number of intracellular progeny has reached 100 (French *et al.*, 1952; Watson and Maaløe, 1953;



Hershey, 1953a). At no time during the latent period does there appear to exist an important fraction of the parental DNA which forms part of structures intermediate in size between free DNA fibers and mature bacteriophage particles (Watanabe *et al.*, 1954). The fact that the parental DNA persists as free nucleic acid and that it, like all other viral DNA precursors present in the host cell at early stages of infection, reappears preferentially in the first progeny phages to mature, leads one to infer that the transferred parental DNA simply forms part of the general intrabacterial pool of viral precursor DNA already discussed in previous sections.

## 2. *Distribution of Transferred Material*

Although the experiments just described point to the fact that most of the transferred parental DNA atoms remain part of specific polynucleotides throughout the intracellular reproductive processes, they do not permit any conclusions as to whether the *integrity* of the parental phage DNA is really conserved. That is, do the DNA atoms transferred from one parental virus all wind up in a single progeny particle or are they dispersed over many of the descendants? It has been the hope that the answer to this question might give some clue to the mechanism by which the parental phage DNA goes about its own manifold reduplication, since an important operational distinction between different conceivable DNA replication schemes is their prediction of the distribution of the atoms of the parental molecule over the replica structure (Stent, 1953; Delbrück and Stent, 1957).

The problem of the distribution of the transferred DNA atoms has been solved by two rather different techniques, each capable of measuring the  $P^{32}$  content of individual virus particles in a heterogeneously labeled population. The more direct of these techniques, an autoradiographic method developed by Levinthal (1955, 1956), involves measurement of the  $P^{32}$  content of individual phages by embedding the radioactive particles in a sensitive photographic emulsion, allowing radioactive decay to proceed, and counting the number of  $\beta$ -ray tracks found to be emanating from point sources after development of the exposed emulsion. Since each track represents the decay of a single  $P^{32}$  atom, the total  $P^{32}$  content of each source can be calculated from the number of tracks and from the exposure time and known decay rate of the isotope. A second, much less direct method, measures the  $P^{32}$  content of the individual phages by observing the lethal effects of the decay of the radioactive atoms on the particle in which the decay has taken place (Stent and Jerne, 1955; Stent *et al.*, 1959). Since one out of every ten such  $P^{32}$  disintegrations is lethal (Hershey *et al.*, 1951a), the rate of death with radioactive decay of individual particles is proportional to their content of the labeled isotope. Therefore, in order to measure the distribution of the transferred parental DNA, bacteria are infected with highly  $P^{32}$ -labeled

phage particles and individual progeny particles are examined for their content of radioactivity by either one of these techniques. Such experiments show that the integrity of the parental DNA is not conserved in the course of the reproduction process and that, instead, the transferred radioactive parental atoms are dispersed over a number of the descendants. The distribution of the parental label, however, is far from uniform, in that about half of the transferred atoms wind up in progeny particles which harbor as much as 20 % of the DNA of the parent phage, whereas the remaining half of the transferred atoms are dispersed into very much smaller fragments. If the first generation progeny are passed through a second cycle of growth and the distribution of the grandparental DNA atoms among the second generation progeny studied, it is found that the second-growth cycle has not produced any serious diminution of the 20 % DNA pieces in either size or frequency relative to the total transferred  $P^{32}$ . Contrary to the high hopes held for them at the outset of these experiments, they do not seem as yet to have brought any decisive revelations concerning the DNA replication mechanism. The reader may, however, find a number of interpretations of their results in the original publications (Levinthal, 1955, 1956; Stent and Jerne, 1955; Stent *et al.*, 1958) and in reviews devoted to the subject (Hershey and Burgi, 1956; Delbrück and Stent, 1957).

### *C. Superinfection Breakdown*

Shortly after the onset of phage development, some change takes place within the phage-infected bacterium which becomes apparent only upon subsequent infection of the same cell by another homologous phage particle. This change was observed when bacteria already multiply infected with nonlabeled T-even phages were superinfected with an inoculum of  $P^{32}$ -labeled T-even particles at various later times. For the result of this experiment was that if more than 3 or 4 minutes have elapsed between primary and secondary infections, then half of the label of the superinfecting phages is liberated into the medium in a low molecular weight, acid-soluble form. If, however, the secondary infection is made immediately after the primary infection, then most of the  $P^{32}$  of the labeled phages remains in a high molecular weight, "acid-insoluble" form (Lesley *et al.*, 1950, 1951). This *superinfection breakdown* of the DNA of the secondary phages appears to be a way of excluding latecomers from participating in the intracellular reproductive processes, since the formation of acid-soluble label under these conditions is exactly correlated with a decrease in both transfer of DNA and genetic contribution from the superinfecting individuals to the progeny phages (Dulbecco, 1952; Graham, 1953). Superinfection breakdown appears to involve digestion of the superinfecting DNA injected into the host cell by an intrabacterial deoxyribonuclease (DNAase), since the breakdown (though not the

exclusion of the superinfecting DNA from transfer or genetic contribution) can be prevented by conditions known to inhibit the action of this enzyme (French *et al.*, 1952). It is not unlikely that superinfection breakdown is related to the general increase in DNAase activity which occurs in T-even infected bacteria (Pardee and Williams, 1952, 1953) and which represents the phage-induced destruction of a bacterial ribonucleic acid inhibitory to the action of an endogenous bacterial DNAase (Kozloff, 1953). The significance and possible role of superinfection breakdown and increased DNAase activity for vegetative bacteriophage multiplication still remain to be elucidated, however.

## VI. COMPARATIVE BACTERIAL VIROLOGY

Before closing this account of the biological and chemical sequence of phage multiplication, it must once more be recalled to the reader that practically all of the experimental evidence cited in the preceding sections has concerned only one restricted, and from many points of view, rather peculiar group of bacterial viruses, the T-even strains active on *E. coli*. Surely, it is presumptuous to have spoken so frequently of "the bacteriophage" when, in fact, only the T-even group could have been properly meant, and when the applicability to bacterial viruses in general of this or that inference may be either unsupported by any direct evidence or, worse yet, even be contradicted by experience. The fact is, however, that with no other type of bacteriophages has there been carried out an amount of chemical and biological work comparable to the effort already expended on the T-even strains. Any attempt to present an integral view of phage multiplication must, therefore either be blithely one-sided in favor of the T-even strains or else be saturninely interspersed with obscurantist reminders of the existence of other less-known systems. Nevertheless, we shall glean the ground of the preceding sections for a brief appreciation of similarities and differences between the T-even strains and those other phage types on which some relevant information is available.

First of all, the essential features of the one-step growth curve appear to be the same for all bacteriophages which have been examined from this point of view, i.e., a latent period of constant titer, followed by liberation of a burst of progeny particles after lysis of the host cell. Likewise, the eclipse phenomenon has been observed with all those bacterial viruses where it has been sought. The total disappearance of infectivity from an infected cell at early stages of the latent period, furthermore, is also encountered with those animal viruses for which techniques of study comparable to those of bacteriophage experimentation are available (Dulbecco, 1955). In fact, the existence of a non-infective eclipse phase during their life cycle is perhaps that attribute of viruses which can serve most readily to distinguish them from other obligate intracellular parasites (Luria, 1950).

Incomplete forms of phage proteins have also been detected in bacteria infected with phage types outside the T-even series. For instance, complement-fixing phage antigens are present in phage 3A-infected staphylococci and T5-infected coli in advance of the appearance of any mature progeny, and the total complement-fixing activity present at later times of the latent period is always in excess of that accounted for by infective phage particles (Rountree, 1951, 1952; Lanni, 1954). In the case of T3 phage, however, no *neutralizing* antigens seem to be present in the infected cells either in advance of the appearance of infective progeny or in excess of that accounted for by the mature progeny (Barry, 1954). Empty phage heads, like the T-even doughnuts, have been observed in electron micrographs of lysed coli in which development of phage  $\lambda$  is under way (Kellenberger and Kellenberger, 1957). Also, proflavine interferes with the maturation of phages T1, T3, T5, and T7 in a manner similar to its inhibitory effects on the T-even group (Foster, 1948). Indeed, in the case of some temperate *pyocyanea*, *megatherium*, and coliphages, the effect of proflavine of preventing the maturation of complete phage particles can also be produced by a genetic alteration of the prophage of a lysogenic bacterium. In such "defective" lysogenic strains, phage development can be induced by the usual means (cf. Chap. 9). After a time corresponding to the normal latent period, the defective lysogenic cells lyse and liberate both the genetic material and the protein of the progeny, without yielding any morphologically intact, infective phage particles (Jacob, 1950; Lwoff and Siminovitch, 1951; Lederberg and Lederberg, 1953; Appleyard, 1954; Jacob and Wollman, 1956). Finally, also, phenotypic mixing does not appear to be restricted to the T-even phages, since it has been observed with T1 (Tessman and Ozaki, 1957) and with  $\lambda$  (Appleyard *et al.*, 1956). It appears, therefore, that the notion of spatially separate synthesis of phage nucleic acid and phage protein can be extended with some justification to bacterial viruses other than the T-even group. Unfortunately, however, there is no immediate prospect of studying the intracellular appearance of phage DNA of any strains other than the T-even, or carrying out chemical "pool-size" measurements of phage precursor nucleic acid, since thus far only the DNA of the T-even phages is known to be sufficiently different in composition from the DNA of their host, so that phage DNA can be recognized specifically in the presence of host DNA (Cohen, 1955). For this same reason, it has not yet been possible to ascertain whether or not the fact that protein synthesis inhibitors, such as chloramphenicol, arrest the intracellular multiplication of T1 and  $\lambda$  bacteriophages (Bozeman *et al.*, 1954; Miki and Matsushita, 1956) means that the synthesis of the DNA of these strains also cannot get underway unless the formation of a "nonprecursor" protein is first allowed to take place. (A report that DNA synthesis *does* proceed in bacteria which have been infected in the presence of chloramphenicol with any one of a number of phage strains other



than the T-even group illustrates this difficulty, since it is by no means clear whether this observation really reflects the appearance of phage DNA or only the continuation of host DNA anabolism (Crawford, 1957).)

A few radioactive tracer experiments have been carried out with other phage-host systems. The bacterial contribution to the phage nucleic acid, for instance, has also been studied for strains T1, and for the two related strains, T3 and T7, by removal or addition of a labeled isotope at the moment of infection of the bacterial culture (Labaw, 1951; Putnam *et al.*, 1952). Here, the bacterial contribution is greater than that to the T-even strains, since phage T1 derives about 56 % of its phosphorus, and T3 and T7 from 60-90 % of their phosphorus, from materials already present in the host cell at the moment of infection. Kinetic assimilation studies of the atoms of the bacterial contribution similar to those already described for the T-even strains show that here too, the host nucleic acids are provenance of the bacterial contribution (Labaw, 1953), although the relative importance of the contribution from bacterial RNA and bacterial DNA has not yet been assessed. The bacterial contribution to the DNA of phage T5 has been reported as 30 % i.e., similar to that of the T-even strains (Labaw, 1951). Transfer measurements have also been carried out with  $P^{32}$ -labeled stocks of T3 and T7, which showed that from 30 to 50 % of the parental labeled atoms reappear among the progeny in each case (Kozloff, 1953; Watson and Maaloe, 1953). Hence, also, the transfer of parental nucleic acid to progeny can be considered to be a general phenomenon associated with phage reproduction. It must be noted with regret, however, that at the time of writing, the blender experiment of Hershey and Chase (1952), that great milestone which signaled the germinal role of the phage DNA, has not yet been repeated successfully with any phage outside the T-even group.

There is one aspect, however, in which the T-even phages rather differ from many other bacteriophages; that is the way in which they affect the host cell in the course of their reproduction. Infection with T-even phages immediately arrests bacterial growth, as measured by either the turbidity or rate of respiration of the infected culture (Cohen and Anderson, 1946), and suppresses formation of induced bacterial enzymes (Benzer, 1953). (Such interference with induced enzyme synthesis was actually first discovered by Monod and Wollman (1947) with another coliphage, and later also found to obtain with the pyocyanea phage P2 (Jacob, 1951).) Intracellular multiplication of phage strains, like the pyocyanea phage P8 (Jacob, 1952) and the *megatherium* phage 899 (Siminovitch and Rapkine, 1952; Siminovitch and Jacob, 1952), does not, on the other hand, produce such drastic effects and allows the infected bacteria to increase in size and synthesize respiratory as well as induced enzymes. Cytological examination of phage-infected bacteria reveals that while growth of the T-even phages quickly disrupts the host cell nucleus, the

reproduction of other phage strains, such as T1, T7,  $\lambda$ , P1, and P2, leaves the nucleus largely intact throughout most of the latent period (Luria and Human, 1950; Murray *et al.*, 1950; Murray and Whitfield, 1953; Kellenberger, 1953; Whitfield and Murray, 1954). It is conceivable that the previously mentioned increase in intrabacterial DNAase activity and its possible epiphenomenon, superinfection breakdown, are related to the dissolution of the host nucleus in T-even, infected cells, since there is neither any increase in DNAase activity (Pardee and Williams, 1953; Wormser and Pardee, 1957) nor any establishment of superinfection breakdown (French *et al.*, 1951) in bacteria infected with T1, T3, T7, or  $\lambda$ , i.e., with phages which permit the nucleus to remain intact. Most likely, it is the destruction of the host cell nucleus by the T-even phages which is responsible for their suppression of cellular growth and enzyme induction, since the integrity of the nucleus appears to be essential for bacterial enzyme synthesis (McFall *et al.*, 1958). Some other striking differences in behavior between the T-even phages and various phage strains have led to the following notion<sup>1</sup> (Stent, 1958): The T-even phages are characterized by high UV sensitivity and high multiplicity reactivability of the free virus particles, low radiosensitivity of the "capacity" of the bacterial host, and frequent genetic exchange between vegetative phages in multiple infection. They can, therefore, *dispense with* the integrity of the host cell nucleus, which they destroy anyway at the outset of their growth. A second group of phages, which includes T1, T3, T7,  $\lambda$ , and P22, are characterized by low UV sensitivity and low multiplicity reactivability of the free virus particles, high radiosensitivity of the capacity of the bacterial host and even higher radiosensitivity of the capacity for the reproduction of irradiated phages, existence of UV reactivation, and its attendant appearance of new genotypes and infrequent genetic exchanges between vegetative phages in multiple infection. This second group, therefore, *requires* the integrity of the host cell nucleus, with which these phages undergo repeated genetic interactions in the course of their vegetative growth. This same idea might also account for the *exclusion* of T1, T7, or  $\lambda$  from bacteria simultaneously infected by a T-even particle (Delbrück and Luria, 1942; Luria and Delbrück, 1942; Delbrück, 1945b; Weigle and Delbrück, 1951), since phages like T1, T7, or  $\lambda$  should not prosper in a cell in which the presence of a T-even phage has already destroyed the bacterial nucleus necessary for their reproduction.

One important type of intracellular bacterial virus multiplication has been almost entirely neglected in this chapter, namely, that ensuing not from infection by an exogenous virus particle, but from induction of a prophage within a lysogenic cell. This process is discussed in Chapter 9 of this volume,

<sup>1</sup> The phenomena referred to here are actually outside the province of the chapter and are elucidated in Chapters 8 and 10, Vol. II.

and we shall state here only that once the prophage has given rise to a vegetative phage, the sequence of events in the induced lysogenic bacterium is quite similar to that in a sensitive bacterium infected with the same bacteriophage strain (Lwoff, 1953).

## VII. THE VEGETATIVE PHAGE

We have had frequent occasion to speak of *vegetative phage* in the preceding, without having bothered to bring this concept into focus. But some difficulties present themselves now in connection with this notion, which are not unlike those which eventually developed with the meaning of the term *gene*. After gene had long served as the unit of recombination, mutation, and function, it became necessary to re-examine the idea of the unit of inheritance, when the study of genetic processes revealed that these three aspects of the hereditary substance are by no means inseparable (Pontecorvo, 1952; Benzer, 1957). Similarly, after "vegetative phage" had first represented simply the non-infective form in which the bacteriophage multiplies within the host cell, it later transpired, as we have seen, that there is no one "form," but rather that a variety of different phage-related precursor and nonprecursor structures make their appearance within the infected bacterial cell. Hershey (1957a) proposed, therefore, that the meaning of the term vegetative phage should be restricted to "the physical structure with which the new germinal substance is associated during vegetative reproduction," or to what Lwoff (1953) had already called the "gonophage." This restriction eliminates the incomplete phage proteins, such as the precipitable and serum-blocking antigens or the doughnuts discussed in Section III, from the vegetative phage concept, since these materials pertain to the somatic rather than germinal substance of the virus, and leaves the field clear for the phage precursor DNA, the presumptive carrier of the hereditary information of the extracellular phage particle. Nevertheless, even this more precise definition is not unambiguous, because the general nature of the mechanism by which the phage DNA is synthesized is not yet understood. The ambiguity becomes apparent if we consider two possible alternative DNA replication models.

Model 1. The DNA molecules injected into the host cell by the parent phage reduplicate by serving *directly* as the "template" for the ordered copolymerization of replica DNA molecules of nucleotide sequence identical to the parental molecules, possibly by means of the mechanism of complementary base pairing suggested by Watson and Crick (1953) or one of its numerous subsequent modifications (cf. Delbrück and Stent, 1957). Each replica DNA molecule in turn serves as the template in further replication cycles, and hence the entire process is geometric. Under this mode, the vegetative phage is the phage precursor DNA.

Model 2. At the outset of the infection process, the parental DNA molecules *transfer* their hereditary information to some other type of macromolecules, which then in turn act as templates in the synthesis of replica phage precursor DNA molecules. If only the parental but not the replica DNA molecules act in the establishment of these templates, then the entire process of DNA replication is linear. If, on the other hand, there is a turnover of templates and both parental as well as replica DNA molecules continue to transfer their information and establish templates, even at late stages of intracellular phage growth, then the process of DNA replication is quasi-geometric. Under this model the germinal substance of the phage is "associated" with *both* the phage precursor DNA *and* the non-DNA templates during vegetative reproduction, and hence it is not clear upon which structure the name vegetative phage should be conferred.

That the process of replication of the genetic substance, and, by inference, of the precursor DNA of the bacteriophage is geometric is indicated by an experiment of Luria (1951), which showed that the distribution of spontaneous phage mutants in single infected bacteria is clonal (cf. Chapter 8). This finding, however, does not distinguish between the two models. Model 2 finds support from two different directions. If highly  $P^{32}$ -labeled bacteria are infected with equally highly labeled T-even phages and the lethal effects of  $P^{32}$  decay followed at various stages of the latent period, it is found that radioactive disintegrations can no longer prevent the appearance of infective progeny if phage development has already proceeded for 7 or 8 minutes, even though both the infecting DNA as well as any of its vegetative replicas which may have already been formed are highly radioactive and ought to be subject to destruction by radioactive decay (Stent, 1955). Model 2 explains this unexpected observation, if the non-DNA templates to which the hereditary information is transferred are not sensitive to disruption by disintegration of incorporated  $P^{32}$  atoms, either because they contain no phosphorus (protein?) or because they possess a structure of greater inherent stability than the free DNA duplex (nucleoprotein?). Second, the role of the essential nonprecursor protein discussed in Section IV, B, whose synthesis during the first few minutes after infection must be allowed to occur before the formation of phage precursor DNA can take place is also readily accounted for by Model 2, if this protein forms an integral part of the non-DNA template. In fact, a Model 2-type molecular scheme of the replication and genetic recombination of the hereditary material of the phage has been conceived in which the templates are ribonucleoprotein molecules made up of the postinfection RNA and the essential nonprecursor protein of Section IV (Stent, 1958). The experimental resolution of the question of the chemical nature of the vegetative phage and its mode of replication and genetic exchange is one of the immediate goals of present research efforts.



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## Chapter VIII

### Bacteriophage Genetics

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#### I. INTRODUCTION

Phage genetics is at present the subject of a rather profound disagreement among the workers in the field. The disagreement is not about experimental results and is only partly connected with the direct interpretation and calculation of the results. It is more a disagreement about the prospects for the immediate future. There are some who feel that most of the essential results

can be encompassed in a reasonably coherent and simple picture and that the main effort will involve further attempts to understand the details of the formal genetic schemes in terms of the physical chemistry of the DNA molecule. On the other hand, there are others who see in some of the current problems and apparent contradictions, the prospect of a breakdown of most of the currently fashionable ideas and, possibly, even a new and fundamental complementarity or uncertainty principle in biology. This chapter will be written from the former point of view. It will also be written for readers who are assumed to be unfamiliar with genetics, microbial or otherwise; and in an attempt to avoid discussions of terminology, the words "gene" and "chromosome" will not be used in connection with phage.

## II. PHAGE MUTATIONS

### *A. Plaque-Type Characters*

If an excess of sensitive bacteria is mixed in a layer of nutrient agar with a small number of phage particles, clear holes or plaques are formed in the otherwise continuous layer of bacterial growth (see Adams (1950) for a discussion of currently employed techniques). If the phage particles in a single plaque are used to inoculate a culture of sensitive bacteria, one can obtain a stock of  $10^{11}$  to  $10^{12}$  particles per milliliter. Most of the plaques produced when this stock is plated are identical with those used for inoculation; but among several thousand, a few will differ in various ways from the majority type. Some of the altered plaques are small, due to a chance delay in the initial attachment of a phage particle to an indicator bacterium. If these are picked and the phage they contain replated, the platings show the same distribution of types that was shown by the original stock. However, an occasional altered plaque is due to a hereditary change in the phage, as can be demonstrated by the fact that the alteration continues to show itself when phage from the plaque is replated. These observable changes in plaque morphology which continue to appear after the modified phage has been grown through several cycles in new bacteria are defined as genetic changes in the phage.

The ability to detect a particular genetic variation may depend on the type of bacterial cells and the medium on which the phage is grown. However, from the point of view of formal genetics one need not consider the nature of the physiological changes in the altered virus or the mechanism by which the observable alteration arises. All that must be determined is that the observable change in plaque morphology continues to show itself when the progeny of the original altered phage are plated. The total number of different observable changes that has been used in phage genetics is small,

probably not more than ten or twenty. However, thousands of different mutations have been used in the various genetic studies carried out with this organism. The many mutations are separately arising inherited alterations which affect one of this small number of observable characteristics.

Most of the detailed genetic analyses have been done with the T phages, which are active on *Escherichia coli*, and in particular the T-even members of this group. The wild or standard type of these phages is defined as the type which was first isolated for experimental purposes (Demerec and Fano, 1945), and strain B of *E. coli* has most commonly been used as indicator for them. The wild-type phage produces a plaque of 1 to 2 mm. in diameter, with a small clear center in which all the bacteria are destroyed and a wider region in which the destruction of the bacterial layer is only partial and gradually diminishes to zero. If a large number of such plaques are examined, one finds that approximately one in ten thousand are larger and have a sharp edge delimiting the area of bacterial clearing. If these altered plaques are picked and diluted, they are found to contain about  $10^7$  particles, all of which produce the same altered plaques. The phages which produce these altered plaques are called "*r*" (for rapidly lysing) mutants (Hershey, 1946a,b); their physiology is discussed in Chapter 7 in connection with the phenomenon of lysis inhibition (Doermann, 1948). Using strain B as indicator, only one type of *r* mutant can be distinguished by the appearance of its plaque; however, if different strains of bacteria are used, three different classes of *r* mutants can be recognized (Benzer, 1957) by their appearance and by the fact that some of them fail to produce any plaques on one or more of the different indicators.

Another set of directly observable mutations are the *tu*'s (turbids) used by Doermann and Hill (1953) in studies with the phage T4. The plaques produced by these phages have a turbid ring like the *r*<sup>+</sup>, but the outer edge of the ring is sharp and distinct. Under the proper conditions of plating, one can determine by direct observation if a plaque was produced by a phage of the type *rtu*, *rtu*<sup>+</sup>, *r*<sup>+</sup>*tu*, or *r*<sup>+</sup>*tu*<sup>+</sup>. In this and in the discussion of other mutants we shall follow the standard genetic notation and use the symbol *r*<sup>+</sup> to mean "like the wild type with respect to *r*" and *tu*<sup>+</sup> to mean "like the wild type with respect to turbid."

If certain indicator dyes are added to the agar, some phage mutants will produce plaques in which there is a material which affects the color of the dye. These "color mutants" of the phage T1 have been used to great advantage and with considerable esthetic appeal by Bresch (1953) in studies of T1 genetics. And finally, to complete the list of directly observable plaque-type mutants of the T phages, there is the minute (*m*) mutation (Hershey and Rotman, 1949), which produces small plaques when it occurs in T2 or T4. It should be kept in mind that this is not in any sense a complete list of the



possible alterations which could be observed as plaque-type mutants. It seems likely that many others could be found if sufficient ingenuity and effort were devoted to establishing plating conditions on which the alterations could express themselves.

### *B. Host-Range Mutants*

All the mutations mentioned so far are observable using *E. coli* strain B as the plating bacterium. If different strains are used, they may enhance or decrease the observable difference. However, there is another type of mutation which can be observed if bacterial mutants are specially selected for this purpose. By mixing a very large number of wild-type phage T2 and many bacteria in a layer of nutrient agar, T2-resistant bacteria, designated B/2, can be selected which will grow even in an excess of phage. If these resistant bacteria are used as indicator, the wild-type phage will not produce plaques; but if many wild-type phages are plated on them, it is possible to select phage mutants which are able to grow and produce plaques on the resistant bacteria. These host-range mutants, which are designated *h*, are discussed more fully in the chapter on adsorption, since the genetic character expresses itself by its effect on the ability of the phage to adsorb to the resistant cells. Using mixed indicator, which contains both the sensitive and resistant cells, one can determine by direct observation whether the phage producing a particular plaque was *h* or *h*<sup>+</sup>. The *h* phage will produce a clear plaque, since it can destroy both types of cells, while the *h*<sup>+</sup> phage will produce a plaque which is turbid because it is overgrown by the resistant cells. This process of selecting resistant bacteria and using them to obtain phage mutants with increased virulence can be repeated and has been used by Baylor *et al.* (1957) to obtain two steps beyond *h* for the T2 system, and by Fraser and Dulbecco (1953) to obtain a set of T3 mutants with numerous levels of extended host range.

Several of the systems of mutations will be discussed below in connection with some of the special problems for which they have been particularly useful. However, it is worth noting that the sets of mutations which are observable depend very much on the particular type that was originally selected as the standard. For example, if one had started the study of T2 genetics with an *h*-type phage and the bacterium B, it is probable that the B/2 and *h*<sup>+</sup> mutation would never have been obtained, since one needs the mutation to B/2 in order to recognize the existence of an *h*<sup>+</sup> phage as different from the *h* and one needs the existence of the *h*<sup>+</sup> phage to detect the presence of a resistant bacterium.

Each of the mutations discussed so far behaves as a single-step process. However, one can obtain phages which differ from the wild type by several mutations if particles mutant for one property are first selected and a large

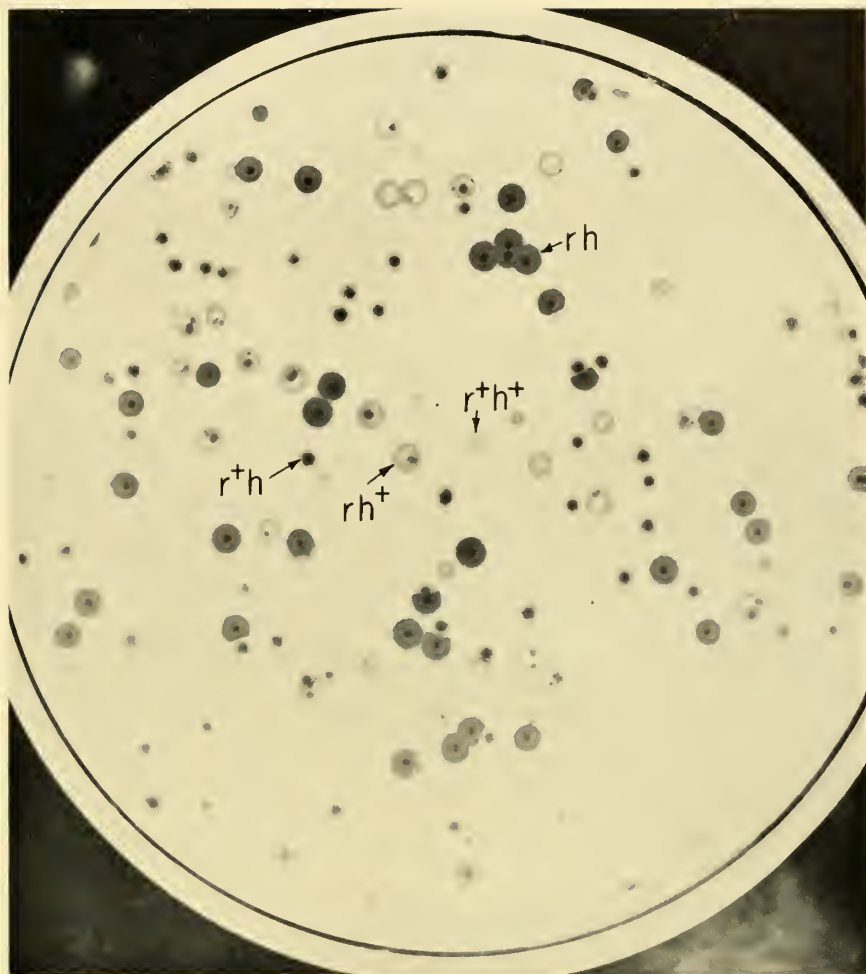


FIG. 1. A photograph of a petri dish on which a mixture of four types of T2 phage  $r^+ h^+$ ,  $rh^+$ ,  $r^+ h$ , and  $rh$  were plated on a mixture of *E. coli* B and the T2 resistant strain B/2. The clear spots in the  $rh^+$  plaques are due to phage mutations to  $h$  which occurred in the plaque.



number of mutant phage are examined for further mutations to other altered properties. Thus, one can obtain an  $rh$  phage if one first selects an  $r$  mutant and then uses the resistant bacteria to select the  $h$  mutation. The phage so obtained will have the plaque morphology of the  $r$  and the host range of the  $h$ . By plating on mixed indicator all four possible combinations of  $r$  and  $h$  can readily be detected. These four types are indicated in Fig. 1.

### III. RECOMBINATION

#### *A. Linkage Map*

The characteristics of an individual phage particle which are directly observable are called the phenotype of that particle. Although the number of different phenotypes is small in the phage system, it is possible to demonstrate that independent mutations, even if they produce the same phenotype, are rarely identical. This difference in the genetic properties can be demonstrated by making a cross between two different phages and observing new recombinant types which have properties different from either parent.

The first paper (Hershey and Rotman, 1948) in which the results of a true genetic analysis were presented concerned the linkage between a number of  $r$  mutations, all of which produce the same phenotype. In this and two succeeding papers, Hershey and his collaborators (Hershey and Rotman, 1948; Hershey and Chase, 1951) presented most of the basic facts and much of the currently accepted theoretical interpretations about the mechanisms involved in phage genetics. In addition, fundamental questions were posed, which the work of many investigators during the following decade has only partially answered.

This first paper demonstrated that linkage structures exist on which mutations can be arranged in a linear sequence. The experimental evidence for the one-dimensional nature of the genetic map in phage has since been extended considerably by Doermann and Hill (1953) for the phage T4 and by Benzer (1957), who has studied a small region of the T4 genetic structure very intensively. This linear mapping of mutants, which can be carried out in phage as well as in higher organisms, is probably the single most important conclusion from any genetic analysis; for this reason the principle will be discussed in some detail.

If one considers four independently arising mutations, which will be indicated as  $a$ ,  $b$ ,  $c$ , and  $d$ , there are six possible ways in which these mutants can be used in a two-factor cross. Suppose that of these six possible pairs, the cross with  $a$  and  $b$  produces more recombinants than any of the others. The two mutations in this pair are taken as the ones with the greatest distance between them. Then the mutation  $c$  is placed between  $a$  and  $b$  in proportion



to the frequency of recombination obtained in the crosses  $a \times c$  and  $b \times c$ , and  $d$  is likewise located by means of the crosses  $a \times d$  and  $b \times d$ . So far the mapping can be done for any arbitrary set of numbers obtained from the crosses; but the one additional cross,  $c \times d$ , determines the consistency of the mapping on a one-dimensional structure. If the independent crosses with  $a$  and  $b$  locate  $c$  and  $d$  near to each other, then one must observe a low recombination frequency in the cross  $c \times d$  if a one-dimensional mapping is to suffice to represent the data.

The ordering of mutations can also be done in a way which is mathematically independent of the determination of distances—by making use of a cross of two particles which differ with respect to three mutations. In such a cross, there will be particles in the progeny which receive one genetic marker from one parent and two from the other. The character that lies between the other two will occur least frequently in the progeny associated with the two markers from the other parent. If any number of different mutations are studied, they can, of course, be ordered along a line by a succession of three-factor crosses. Although these two methods of ordering mutations along a line are independent of each other in a formal sense, all the models which have been suggested to explain one also explain the other. It must, however, be kept in mind in what follows that although the genetic map is defined by a set of real operations, the map itself is an abstract way of representing the results of certain experiments and, except by inference, has no physical reality.

### *B. Techniques of Phage Crosses*

A phage cross is carried out by infecting a single bacterium with two or more different types of virus particles. When the phages are mixed with bacteria in a liquid suspension, the rate at which they attach is determined only by the bacterial concentration and the composition of the medium. If care is taken to use bacteria which are equally sensitive to each of the infecting phage types, one can obtain cells in which two or three genetically different types grow. The number of each of the phage types which take part in the infection is determined by the proportions of the various types added to the absorption tube. In preparing these mixedly infected cells, it is frequently necessary to use some method of arresting the development of the system so that the first phage particle to attach does not prevent late arrivals from participating in the growth process. The way in which the first phage particle to infect a cell can exclude others which arrive a few minutes later is not clear, but the phenomenon can be eliminated by agents which temporarily stop cell metabolism. After a sufficient amount of phage has adsorbed, the unadsorbed parental particles must be eliminated, either by differential centrifugation or by antiphage serum; the infected cells are then

transferred into a growth medium and left until the progeny are produced. Except for the controls necessary to determine the multiplicity of infection, these steps are the only ones necessary to carry out a cross.

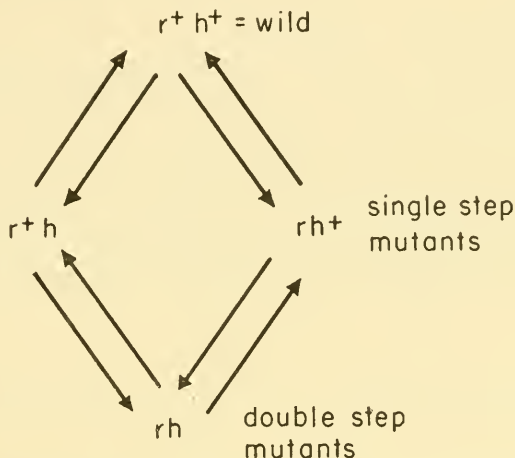


FIG. 2. The mutational pattern of a T2 or T4 phage. If one starts with the wild or standard type, designated  $r^+ h^+$ , single-step mutants can be obtained either to  $h$  or to any of the many genetically different  $r$ 's. A second mutation can then produce the  $rh$  type. However, the  $rh$ 's produced by the two different pathways or on different attempts will in general not be genetically identical. Although all of the steps are reversible, the frequencies are extremely variable and the frequencies for the reversions are very different from one mutation to the next.

### C. Two-factor Crosses

If either a multiple or a single infection is carried out using only the phage in one stock for the parental particles, no new types are found in the progeny except for the occasional mutants. The same is true if two different phage types mixedly infect a single cell, as long as the two types differ with respect to only a single mutation. If, however, two different types of mutant phage, for example, an  $r$  and an  $h$  are used, then some particles appear in the progeny which are like wild type in having neither the  $r$  nor the  $h$  property, and other particles are formed which have both the  $r$  and the  $h$  (Fig. 2). These  $rh$  particles behave in the same way as those obtained by successive selection of the double mutants. Likewise, if two stocks of  $r$  mutants which arose independently are crossed in the same way, wild-type particles are also observed in the progeny. However, the phage particles containing both the  $r$  mutations can be identified only by the fact that if they are further crossed to either of the parenteral  $r$ 's individually, no wild-type plaques are observed. On the

other hand, if they are crossed to the wild type, some of the progeny then behave in further crosses as though they contained only a single  $r$  mutation. In general, a phage with only a single  $r$  mutation will give wild-type recombinants when crossed to one of the original  $r$  stocks but not with the other, whereas a phage containing the double  $r$  will not give such recombinants when crossed with either.

In a cross  $ab \times a^+b^+$  the number of recombinants of the type  $ab^+$  is generally equal to the number of recombinants of the type  $a^+b$ . This conclusion is not dependent on the nature of the mutations, i.e., the symbols  $a$ ,  $b$ , etc., can represent any mutations which arise independently. In addition to the equal numbers of the reciprocals in such a cross, one also finds the same total number of recombinants if the two mutations are in one of the parents or if each mutation is introduced into the cross in a different parent. Thus, the last two crosses in Table I would always yield the same number of recom-

TABLE I  
VARIOUS CROSSES AND THEIR RECOMBINANT TYPES

| Cross                       | Progeny            |  |
|-----------------------------|--------------------|--|
|                             | Parental Types     | Recombinant Types                                      |
| $r \times h$                | $r, h$             | $rh$ , wild  |
| $rh \times \text{wild}$     | $rh$ , wild        | $r, h$   |
| $r_a \times r_b$            | $r_a, r_b$         | $r_a r_b$ , wild                                       |
| $r_{ab} \times r_a$         | $r_{ab}, r_a$      | none   |
| $r_{ab} \times r_b$         | $r_{ab}, r_b$      | none   |
| $r_{ab} \times \text{wild}$ | $r_{ab}$ , wild    | $r_a, r_b$   |
| $ab^+ \times a^+ b$         | $ab^+, a^+ b$      | $ab, a^+ b^+$  |
| $ab \times a^+ b^+$         | $ab, a^+ b^+$      | $ab^+, a^+ b$  |
| $abc \times a^+ b^+ c^+$    | $abc, a^+ b^+ c^+$ | $a^+ bc, a^+ b^+ c, a^+ bc^+, ab^+ c, ab^+ c^+, abc^+$ |

binants. These equalities do not, however, reflect any particular property of the elementary mating act. They indicate only that none of the phage types has any growth advantage relative to the others and that the nature of the mutants does not affect the probability of the recombinational event occurring. Thus, in the cross  $ab \times a^+b^+$ , the number of progeny particles carrying the genetic property  $a$  should be equal to the number of those carrying  $b$  if there is no selection. This implies that in the progeny the following relationship must hold

$$ab + ab^+ = ab + a^+b$$

or

$$ab^+ = a^+b$$

where the symbols now represent the *number* of particles of the various types in the progeny.

If we let the ratio of the two types of parental particles in the mixedly infected cell be

$$\frac{p^1}{p^2} = \frac{ab}{a^+b^+}$$

then it also follows from the lack of selection in the system that

$$\frac{ab + ab^+}{a^+b^+ + a^+b} = \frac{p^1}{p^2}$$

And, finally, the sum of all the types in the progeny is identical with the burst size (*B. S.*), which is the mean number of progeny liberated per infected cell; in a standard cross the burst size always has the same value. Thus

$$ab + a^+b^+ + a^+b + ab^+ = B. S.$$

We can conclude from the foregoing that the results of any cross between two markers can always be described by one number. This follows from the fact that there are four numbers to be measured and three equations which relate them. The measured parameter which describes the cross is usually taken as the frequency of recombination. In the above cross this would be

$$\frac{a^+b + ab^+}{B. S.} = R_{ab}$$

#### *D. Three-Factor Cross*

If a standard cross is made between two phage stocks which differ from each other with respect to three mutations, the situation is a little more complicated. In the cross  $abc \times a^+b^+c^+$ , there will be in addition to the parental types six types of recombinants produced in the progeny. However, there are certain general rules which yield relationships between these eight types. The total number of phages which contain the mutation *a* is the same as the total number which contain the mutation *b* or the mutation *c*. Using the symbol  $\cdot$  to indicate either the mutation or the wild type, we can write these relationships as  $a \cdot \cdot = \cdot b \cdot = \cdot \cdot c$ . As before, the sum of all eight types is constant and equal to the burst size, and in addition the ratio of *a* to *a*<sup>+</sup>, or *b* to *b*<sup>+</sup>, or *c* to *c*<sup>+</sup> must be the same and equal to the ratio of the two types of phage among the parents, that is,  $p^1/p^2$ . Thus, if one knows the ratio of the two parental types which went into the cross, one has now four equations which relate the various measured quantities in the progeny; and therefore there are four independent parameters which can be determined in any experiment. Three of these numbers represent the recombination



frequency between the three pairs of genetic markers which would have been obtained if the three two-factor crosses which are possible with the markers *a*, *b*, and *c* had been carried out. The fourth parameter measures the probability that a particle which has had a recombination between two of the markers also has a recombination between the other two. It is the first three of these parameters, namely, the three distances, which allow one to determine the order of the markers along the genetic map (see Fig. 3) and the

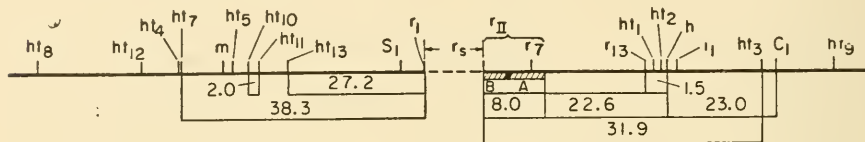


FIG. 3. A linkage map of the phage T2 with most of the known markers indicated; *r1*, *r7*, *r13*, *m*, and *h* from Hershey and Rotman (1948); *ht6* through *ht13* from Baylor *et al.* (1957); the *c* region contains at least three different mutable sites (Brenner, 1957); *i1* (McFall and Stent, 1958) is a mutation which suppresses the action of *h1*, an extended host-range mutant studied by Baylor *et al.*; *S1* is a star-producing mutant which produces slow growth under some plating conditions (Symonds, 1958); *rs* (McFall and Stent, 1958) is an apparent "deletion" situated between the distantly linked *r7* and *r1*; in the *A* and *B* cistrons of the *rII* region, approximately 2,500 independently arising mutants have been mapped (Benzer, 1957).

additivity of distances along this map, while the fourth parameter is a measure of the *interference* between two adjacent recombinations. This latter point will be discussed further below.

#### IV. MULTIPLE MATINGS IN INDIVIDUAL CROSSES

If a cross is made in which three different parental mutants are used to infect the same bacterium, progeny particles are observed (Hershey and Chase, 1951) in which all three mutations are combined in the same particle. One can then conclude that if the elementary mating act involves the combination of only two particles, repeated matings must take place in the cell, and the recombinants formed in one event must be able to enter into other mating events. This conclusion is supported by the fact (Doermann and Hill, 1953; Visconti and Delbrück, 1953) that in a cross with one parent in great excess over the other more recombinants can be observed than minority parental types. If each particle could only mate once, one would expect to find, at most, an equal number of recombinants and minority parents. In addition to the fact that recombinants can engage in further mating, it is also clear that they are able to multiply, since the frequency of recombinants from a cross increases as the burst size increases with delayed time of lysis (Hershey and Chase, 1951; Levinthal and Visconti, 1953; Doermann, 1953).

Geiringer, in a series of papers (1944, 1945, 1948, 1949), developed the mathematical theory of populations in which such repeated matings occur. She derived the general formula for the situation in which any number of genetically different types interact pairwise for an arbitrary number of generations as well as the more limited special cases which are of particular interest to the biologist. One of the most important aspects of her treatment is that it is completely independent of the nature of the elementary act by which recombinants are formed. It is not necessary to specify how many particles are produced in the mating act or whether the parental types are conserved or whether reciprocal recombinants are formed in the same event.

It is only necessary to determine the over-all probability that a particular genetic marker is separated from its neighbors in one generation. Thus, in a mating event between a particle of the type  $abc$  and one of the type  $a^+b^+c^+$ , the theory makes use of the probability of forming the recombinants  $a^+bc$ ,  $ab^+c$ , and  $abc^+$ ; it assumes that these probabilities are equal to those for forming  $ab^+c^+$ ,  $a^+bc^+$ , and  $a^+b^+c$ . Geiringer shows how these probabilities are related to the more usual genetic parameters giving the probability of recombination between each pair of markers under various assumptions as to the interference between adjacent recombinational events.

Visconti and Delbrück (1953) applied the methods of Geiringer to an analysis of phage genetics, and they independently derived the formula for a cross involving three genetic markers. This formula [Equation 5 (Geiringer, 1945) and Equation 8 (Visconti and Delbrück, 1953)] gives the expected frequencies of each of the possible types in the progeny as a function of the inputs of each of the parental types and four additional parameters, namely, the probabilities that each of the markers becomes separated from its neighbors in one mating and the number of rounds of mating. The three probabilities involved here can then be related to recombination probabilities between pairs of markers if an additional assumption is made as to the interference between adjacent recombinational events.

Symonds (1953) expanded the analysis to show that if the number of matings in which each particle or its ancestors takes part is distributed at random, then for two- or three-factor crosses a simple modification of the Geiringer formulas should represent the results.

Visconti and Delbrück showed that a good fit could be obtained with the experimental data for the phages T2 and T4 by making the following assumptions: (a) there is complete mixing of the genetic structures (which they called vegetative phage) in a mating pool within the infected bacterium; (b) there is repeated pairwise mating of these vegetative phage which find their partners in a random process; and (c) the genetic material of the phage does not separate into individual linkage groups which assort independently of

each other. And, finally, they showed that if the mating act is similar to the crossing-over process which occurs in higher organisms, five rounds of mating are, on the average, necessary to account for the results of a standard cross. In T2, if lysis is inhibited, the mean number of rounds of mating will increase by a factor of six or seven before all the mature phage has been formed; during this increased period of phage production, the burst size rises by roughly the same factor.

The same type of population analysis has been used to interpret the data obtained with the phages T1 (Bresch and Menningmann, 1954; Bresch and Trautner, 1955) and  $\lambda$  (Kaiser, 1955; Jacob and Wollman, 1954, 1955; Wollman and Jacob, 1954) and the same basic assumptions give a good fit with the experimental results if the assumed number of rounds of mating is about one-tenth that in T2. Thus, in these other phages, only about one-half of the particles in the progeny have themselves or their ancestors arisen in a mating act.

The most important result of these analyses is that after all the corrections have been made for multiple matings, the results of all crosses between any markers, except those which are extremely closely linked (see Section VII, D) can be summarized in terms of a single linkage map. The genetic markers can be represented as points along this one-dimensional map, with the distances between markers made proportional to the recombination frequencies corrected for multiple switching events (or cross-overs).<sup>1</sup> Thus, for any three markers, the map distances are strictly additive once all corrections have been made.

In 1958 several groups independently re-analyzed, in more general terms, the problem of multiple matings in order to determine whether the basic assumption of pair-wise mating could be justified by experiment. Stahl and Steinberg, using an essentially new and very powerful, analytical method, were able to show that none of the experiments previously reported could be used to determine the number of particles which enter the mating event, and that no information as to the mechanism of the mating event could, in principle, be obtained from any standard mass culture crosses. Thus a single particle which is produced in a mating could contain genetic information from three or more parental structures. This phenomenon they called *group mating*. Hershey (1958) carried out an extensive study of recombination stimulated by ultraviolet light and concluded that in this case, at least, the results were more easily understood in terms of group than of pair-wise mating.

<sup>1</sup> This correction allows for the fact that if two, four, or any even number of switches (or cross-overs) occur between two markers, there will be no observable recombination. This results in the following expression, giving recombination probabilities  $p$  as a function of map distances  $d$  (Haldane, 1917):

$$p = 1/2(1 - e^{-2d})$$

Bresch and Starlinger (1958) did a similar analysis, and Hausmann and Bresch (1958) performed crosses with three different parental types with which it is possible to distinguish between the various models. They concluded that the number of recombinants which had received genetic markers from each of the three parents was too large to be accounted for by successive pair-wise matings in the infected cell. On the other hand (Trautner and Bresch, 1958), the ratio of recombinants which had markers from three parents to those which had markers from only two varied with time in a way which was not easily understood in terms of group mating. They interpreted the result as a process in which group mating occurs combined with a progressive mixing of parental types which, at the time of infection, they assumed had a tendency to be separated from each other. This question is certainly not yet settled, but, at present, the evidence suggests that group mating can occur.

The mathematical analysis which defines group mating is very general and does not depend on any very specific model. It requires only that several particles can enter a mating event and recombinants can be produced which contain information from all of them. A specific model which is reasonable in view of what is known of other organisms and which is contained in this general definition would be one in which pairing occurred between two structures, but only for a short length of the chromosome. Local pairing could take place between two particles in one region and between one of these and a third elsewhere on the chromosome. Thus repeated local pairing is, formally at least, completely equivalent to group mating as the term is used in these analyses.

Phage S13, the smallest one known, differs from others studied in that it has not been possible with S13 to demonstrate any genetic recombination. Mutants for S13 are known, and attempts to show recombination between them have been made by Zahler (1958) and Tessman and Tessman (1958). Zahler, using both host-range and plaque-type markers, looked for recombinants only in single bursts to make certain that only true cases of mixed infection were scored, and found that the recombination frequencies cannot be greater than  $10^{-3}$ . Tessman and Tessman used a variety of host range markers and were not able to demonstrate any recombination down to the level of  $10^{-7}$ . It is not known if this lack of observable recombination is an inherent characteristic of the phage or if the appropriate conditions for carrying out the cross have not yet been found.

## V. THE MATING EVENT

The question which has provoked the most interest in phage genetics over the last decade was originally posed by Hershey and Rotman (1948) and concerns the nature of the elementary mating act. As it was put by them,



"Are the genetic exchanges reciprocal as one expects for simple cases of crossing over, or must one look for an alternative mechanism more intimately connected with the mode of reproduction of the virus?" Of the various phenomena which have been studied there are three which have yielded most of our meager insight into this problem.

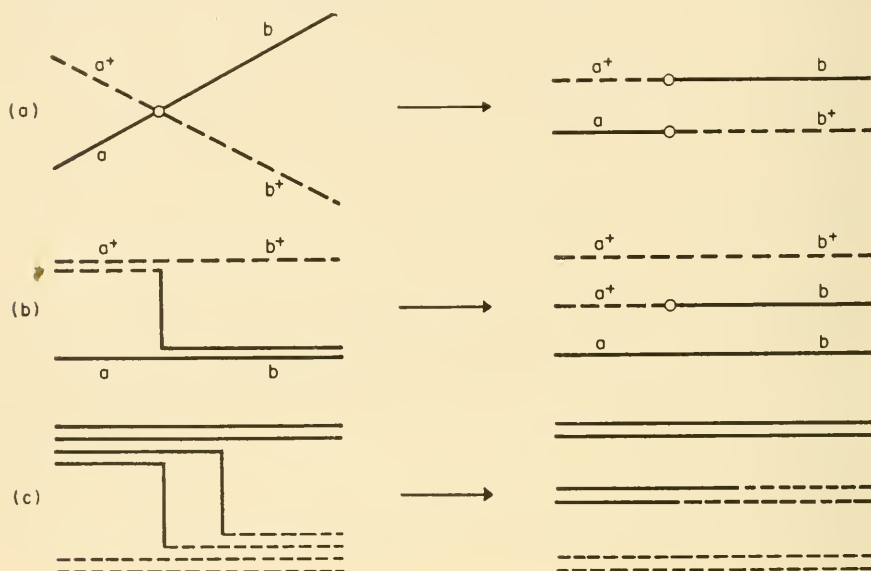


FIG. 4. Three schemes by which recombinants could be formed by the interaction of two parental genetic structures: (a) the classical crossing-over model, which yields two reciprocal recombinants; (b) the partial-replica or copy-choice model, which forms one recombinant and conserves the integrity of the parents; and (c) the partial-replica model, in which all structures are considered as duplex and in which the recombinant contains the heterozygous overlap region in the vicinity of the switch.

### A. Reciprocal Recombinants

As was mentioned previously, the equality of two reciprocal recombinants in any cross is a direct consequence of the lack of selection among the various genetic types. However, in higher organisms, wherever it is possible to perform the required experiments, it is found that the reciprocal recombinants are formed together in the same elementary mating act; largely for this reason, the elementary process in higher organisms is thought of as a crossing-over event. It is, unfortunately, much more difficult to determine whether the reciprocal recombinants arise in the same act in phage than it is, for example, in *Neurospora*, where two pairs of chromosomes enter the mating and all the products of the exchanges are held together in a single membrane

so that they can be examined individually. In phage there is no way of examining the products of an individual mating without at the same time examining all the other phage particles which arose in the same bacterium. Since in any bacterium there are hundreds of independent mating acts, it is difficult to set up an experiment in which one can be sure of examining all the products of a single mating act and avoid the confusion due to different recombinants being produced in different single events.

The problem was investigated in two ways, both of which were first used by Hershey. The experiments involve a modification of the standard procedure of carrying out phage crosses in such a way that the observation is made of the particles liberated by individual, mixedly infected bacteria. The cross itself is done as indicated in Section III, A; but before the infected cells have time to break open and liberate their progeny phage, they are diluted into many individual test tubes to such an extent that each tube has on the average much less than one infected cell. In this way the probability that any tube has more than one cell can be made as small as is necessary. After the cells in this diluted state have been allowed time to produce their progeny, the contents of each tube is examined. Most of the tubes will, of course, contain no phage, while those which do contain any will, for the most part, contain only particles liberated by a single cell. This single-burst technique was originally introduced by Delbrück (1945), and Hershey and Rotman used it to determine whether the reciprocal recombinants produced by single cells were correlated with each other. The correlation coefficient calculated between the per cent recombination of each of the two reciprocal types was not found to be significantly different from that of uncorrelated events. However, the meaning of this lack of correlation was not clear. Since many mating events take place in the cell and since the various recombinants could grow at unequal rates after they were formed, one cannot easily tell whether the lack of correlation is due to the way in which the recombinants are formed or to the events which take place subsequent to the process which formed them. Another method of analyzing the single-burst data gives considerably more convincing results. By using two very closely linked genetic markers, one can obtain a situation in which many of the bursts produce none of a particular recombinant; for these bursts one can ask whether the other recombinant type was produced in its usual amount. In this experiment, also, Hershey and Rotman found that the recombinants seemed to be formed in independent events. It was suggested by Visconti and Delbrück (1953) that this result could also be explained by subsequent growth of one of the recombinants, coupled with a loss of the genetic structures between the time that they are formed in a pool where mating is possible and the time they are wrapped in their protein coats and ready to infect another cell. However, this explanation was made unlikely by Hershey's finding that the

mean number of recombinants of a given type produced by a single cell was considerably greater than unity. This finding seems to be at variance with the idea of a very large loss in the sampling and maturation of the mating particles.

Bresch (1955), in a similar experiment with the phage T1, has been able to supply what seems to be a definitive answer to this question. He used three genetic markers and examined each single burst for all the possible recombinants. Since the number of mating events per bacterium in T1 is very much smaller than that in T2, it is possible to test the correlation under conditions where many of the single bursts produce no recombinant particles of a particular type. A small correlation was observed between reciprocal recombinants, but the same small correlation was also observed between pairs of recombinants which should have no relationship to each other on the basis of crossing over. Thus, the results suggest that in some bacteria conditions are such that the probability of mating is higher than usual, and the frequency of all recombinants is therefore increased. There is no special correlation between the two recombinants that would have been expected had they arisen in a single crossing-over event. The data for T1 showed even more clearly than those for T2 the difficulty of explaining the lack of an observed correlation by a random loss of all phage particles before they were able to produce plaques on indicator bacteria. If a single particle of a given recombinant type appeared on a petri dish, there was a high probability of another particle of the same type being found in the same burst, whereas the probability of finding a particle of the reciprocal type was considerably lower. Were it the random loss of particles which accounted for the loss of an observable correlation, then one would expect to lose the self-correlation as well as the reciprocal correlation. Thus it seems likely that the mating event which forms recombinants in phage is not identical to the process of crossing over which occurs in higher organisms.

### *B. Heterozygotes*

The second phenomenon, which suggests a difference between the recombination event in phage and that in the more classic systems, was reported by Hershey and Chase (1951). They showed that a bacterium mixedly infected with phage particles differing by one genetic marker would produce about two particles in a hundred which behave in a very unusual manner. These particles, called partial heterozygotes, were found in the same frequency for each of the six genetic markers tested; they were characterized by the fact that they produced two different kinds of offspring. The only way these abnormal particles can be detected easily is by means of the mottled plaques they produce when the two kinds of offspring differ with respect to the *r* character. This mottling, which is due to the presence of both the *r* phage

and the  $r^+$  phage in the plaque, can be detected by direct observation. However, in order to detect particles which produce two different kinds of offspring for other genetic markers, it is necessary to allow the plaque to develop, pick it, and retest the phage it contains. Since this process is extremely time-consuming and only a small number of mixed plaques are found, most of the quantitative analyses have been done by selecting plaques which have the observable mottling and testing them for other genetic markers which were introduced in the original cross.

Using the host-range marker  $h$  and various rapidly lysing  $r$  mutants, Hershey and Chase established the following facts about the heterozygous particles: If the  $r$  and the  $h$  which are used in the original mixed infection were separated by at least twenty recombination units (i.e., the recombination frequency is at least 20 %), then most of the mottled plaques, picked and tested for the phage they produced, were found to be pure with respect to the  $h$  marker. In the cross  $hr\ 1 \times h^+r^+$  the mottled plaques would yield either  $hr$  and  $hr^+$  or  $h^+r$  and  $h^+r^+$ . These particles are designated  $hr/hr^+$  and  $h^+r/h^+r^+$ , respectively. However, 6 % of the mottled plaques did yield both  $h$  and  $h^+$ , i.e., they were produced by phage of the type  $hr/h^+r^+$  or  $h^+r/h^+r$ . If an  $r$  was used which was closely linked to the  $h$ , then the situation changed considerably. With  $h$  and  $r13$ , which are two recombination units apart, 75 % of the mottled plaques yielded the two parental types, one of which contained  $h$  and the other  $h^+$ ; the remaining 25 % yielded one parental type and one recombinant, and so were pure with respect to the  $h$  marker.

Using three-factor crosses, Levinthal (1954) showed that if a particle was selected which was heterozygous for the middle of the three markers, that is, it produced mixed offspring with respect to this marker, it was generally recombinant with respect to the outer pair of markers, i.e., its genetic information for the outer markers came from the two different parents. In the cross  $abc \times a^+b^+c^+$  particles selected as being  $b/b^+$  were usually of the form  $abc^+/ab^+c^+$  or  $a^+bc/a^+b^+c$ . Since for the markers used in this case there was a considerable amount of recombination between  $a$  and  $c$  even if the heterozygotes were not selected, it was only possible to show that at least 75 % of the particles heterozygous for the middle marker arose in structures which were recombinant for the outer markers. Trautner (1957), making use of the color mutants in T1, was able to extend this conclusion considerably and demonstrated that over 90 % of the particles in T1 which were heterozygous for a central genetic marker were recombinant for the pair on either side.

These experiments, as well as others performed by Hershey using two  $r$  markers in the original mixed infection, can be summarized pictorially in the following way. We can assume that the phage genetic structure is ordinarily composed of two parallel subunits, each of which contains the same genetic



information. On duplication in a new host these two subunits separate from each other, and each makes another structure which has the same genetic information as itself [as in the model of DNA duplication suggested by Watson and Crick (1953)]. Using this type of model, the picture which we draw for the majority of the heterozygous particles is indicated in Fig. 5 (a). The structures which are shown to be improbable by the three-factor experiments are indicated in Fig. 5 (b). The average length of the overlap region

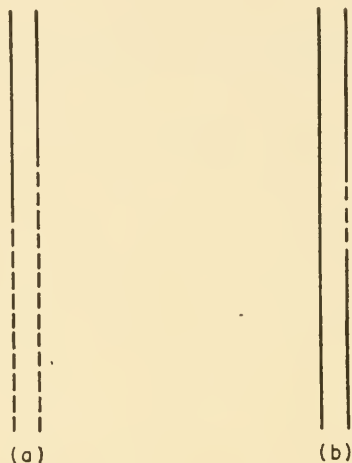


FIG. 5. The two possible schemes by which the two-factor data on heterozygous particles can be represented. The three-factor crosses indicated that the large majority of the observed particles are of the form (a).

can be estimated from the frequency of double heterozygosis for closely linked markers, since this double heterozygosis occurs if both of the markers are on the overlap region. It should be remembered, however, that in drawing a picture of the type indicated in Fig. 5 we are doing no more than indicating graphically by two lines the genotypes of the two segregants or two types of daughters which are usually produced by single heterozygous particles.

The graphic method used here to describe the heterozygotes is sufficient to satisfy all the available experimental data; however, one can certainly invent other models which also satisfy the data. For example, instead of speaking of the average length of the overlap region, with the tacit assumption that the distribution in lengths is more or less normal, one could imagine a situation in which there were a large number of very small overlaps and a smaller number of very large ones. This would also satisfy all the experimental data mentioned above, but would lead to quite different conclusions concerning the role of the heterozygotes in forming recombinants.

In trying to assess the role of the heterozygous particles in phage recombination, one additional experimental finding must be taken into account. If lysis is delayed, a very large burst of phage is produced and in this burst the frequency of recombinants for closely linked markers increases at approximately the same rate as does the burst size. However, during this increase the frequency of mottled plaques remains unchanged (Hershey and Chase, 1951; Levinthal and Visconti, 1953), suggesting that, during the additional growth of phage, heterozygous particles are being lost by segregation into their two daughter types at the same rate at which they are being formed. If the heterozygous particles multiply at the same rate as the rest of the population, then one can show (Levinthal, 1954) that when they segregate they produce most of the recombinants which arise in a cross. This is seen most directly by considering the two particles in a hundred which produce mottling for *r13*. One-quarter of these are pure with respect to the closely linked *h* marker and produce the combinations *hr13* and *hr13*<sup>+</sup> or *h*<sup>+</sup> *r13* and *h*<sup>+</sup> *r13*<sup>+</sup> when they grow in a new bacterium. Thus, the two recombinant types *h*<sup>+</sup> *r13* and *hr13*<sup>+</sup> together comprise one-eighth of the progeny of the *r13* mottlers. This is simply a restatement of the experimental findings with regard to the character of certain mottled plaques; however, if we assume that the heterozygous particles of this same type are being formed and are segregating continuously in the vegetative pool, then they would be a major source of recombinants. For any one generation in the pool this source would add approximately one-quarter of 1 % (one-eighth of 2 %) to the fraction of recombinants between *h* and *r13*, and the several generations in a normal T2 growth cycle would then be sufficient to account for all the 1.5 % of the progeny which are recombinants for these two markers. The weakest point of this analysis is the assumption that the heterozygotes are formed and segregate in the mating pool. One alternative is that they represent some kind of aberration connected with the maturation process. However, if this were the case, it would have to be a very common aberration, since most of the progeny phage contain at least one heterozygous region somewhere in the genetic map. So far it has not been possible to construct a plausible theory as to how these particles could be formed during the maturation.

### *C. Radioactive Tracer Experiments*

In addition to producing reciprocal recombinants in the mating event, the cross-over model also requires the disruption of the particles which enter the mating. The parental sequence of genetic markers no longer exists on a single structure after the crossover has occurred, and likewise the physical structure which contains the genetic information no longer exists as a single entity.

Most of the alternative models which have been proposed involve the formation of recombinants during the replication of genetic material. If replication occurs by the laying down of new material along the parental structure as a template, then the recombinant can be formed if a partially formed daughter switches to copy along another parental structure before it has completed its growth. The recombinant is then a partial replica of one parent combined with a partial replica of the other, and this type of model has been called partial replica formation or copy-choice (see Fig. 4).

One of the consequences of this type of model is the maintenance, both chemically and genetically, of the integrity of the parental structure. It is the chemical integrity of the parental structure which the third type of experiment on the nature of the mating event has been designed to test. For chemical experiments to be relevant to the genetic analysis, one must make certain assumptions as to the nature and the continuity of the carrier of the genetic information. It is known from the experiments of Hershey and Chase (1952) and Hershey (1955) that the genetic information of the phage enters the host bacterium as DNA (deoxyribonucleic acid) and that it leaves the infected cells in newly formed phage as DNA. However, one cannot be sure that the information does not transfer to some other chemical structure during growth in the infected cell. Since there is no compelling evidence to the contrary (see Stent (1958) for a summary of the opposite view), it will be assumed here that the genetic information remains in DNA and, more specifically, that it is the DNA which is involved in the mating events. Thus, if one grows phage labeled in its DNA, one can ask if the label is distributed to the two products of a mating, as would be expected in a crossing-over model, or whether it remains in one particle, as would be expected in a partial replica-type model.

Tracer experiments designed to study the way in which phage particles are produced in the infected cells are discussed in detail in Chapter 7. As was pointed out there, two different techniques (Levinthal, 1955, 1956; Stent and Jerne, 1955; Delbrück and Stent, 1957) have led to the same general conclusions as to the distribution, among single-phage particles, of the  $P^{32}$  atoms which are transferred from labeled parents to progeny. Of the average, about half of the  $P^{32}$  in labeled parental particles is found in the progeny phage after one cycle of growth in unlabeled bacteria. And among the progeny particles there is on the average about one per infected cell which contains approximately 20 % of the  $P^{32}$  in one of the labeled parents. Or, to describe the results differently, about 40 % of the  $P^{32}$  which is transferred to the progeny of each bacterium winds up in a single phage. If a second cycle of growth is allowed in unlabeled bacteria, the total amount of  $P^{32}$  transferred is again about half; thus, a quarter of the phosphorus atoms of the first cycle parents is found in the second cycle progeny. However, the fraction of these transferred atoms

which are still concentrated in single particles remains constant at about 40 %, and the amount of  $P^{32}$  in these particles is still about 20 % of that in the original labeled parents. Thus, one can say that there is a piece of DNA ( $P^{32}$  labels only the DNA of the phage) comprising about 20 % of the total amount in a phage which passes through two growth cycles without being diminished in size, although only about half of the total number of these pieces are transferred in each cycle.

The transfer experiment can be combined with a cross in which  $P^{32}$ -labeled parents containing one set of genetic markers are crossed with unlabeled particles with different markers. If the progeny are first separated according to the genetic markers they contain and then examined for their  $P^{32}$  content, it is found (Levinthal and Thomas, 1957) that phage in the second-cycle progeny, which contain the 20 % piece of DNA, also contain the genetic marker of the labeled parent.

In similar crosses between labeled and unlabeled parents, Garen (1954) showed that not all of the transferred  $P^{32}$  remains with the genotype with which it entered the cross. This result showed either that the genetic structure is broken into several pieces by growth and mating or that only a fraction of the DNA is in a single genetic structure, and the remainder is distributed among the particles of all genotypes. Hershey and Burgi (1956) extended these experiments by varying the relative input of the labeled and unlabeled parent and showed that in a cross followed by one cycle of further growth approximately 40 % of the transferred  $P^{32}$  remained associated with the parental genotype; the remainder was distributed at random. In a further growth cycle the fraction associated with the parental genotype decreased to about 20 %.

These results seem to indicate that there is a piece of DNA in the phage which carries the genetic markers and that this piece of DNA is not broken down by mating. However, there are two reasons why this interpretation is open to question. First, in all the experiments so far reported the separation of genetic types in the progeny has been done only with respect to a single genetic marker—the host range character *h*—which might not be representative of the behavior of the entire genetic structure. In addition to this technical difficulty in the experiments, there is another problem which can affect the interpretation. It is known that the bulk of the  $P^{32}$  which is transferred from parental phage to progeny appears in those particles which are formed early in the latent period (Hershey, 1953), and it is these which have mated the smallest number of times. Therefore, if one looks only at the total progeny in a mass culture, the  $P^{32}$  will be found in those particles which have mated only a few times. It is possible that the observed correlation between the high  $P^{32}$  content and the parental genetic marker is due to the selection of particles which have not in fact mated rather than being a reflection of the



nature of the mating process. This difficulty of interpretation applies to all transfer experiments with phage, and various experiments are now under way to study the transfer in single bursts and with additional genetic markers in an attempt to clarify the point.

## VI. MATING KINETICS

### *A. General Assumptions*

Although it is impossible to eliminate all other alternatives, one can encompass most of the data so far obtained in a self-consistent model of phage growth and recombination. Probably any model which is proposed at this time will have to be modified or extended as further experimental evidence is obtained. It seems useful, however, to formulate in a reasonably precise way the simplest model which at present appears consistent with the available data. To be sufficiently complete to allow detailed predictions to be made, any model must specify two aspects of the processes which occur in the infected bacterium. First, it must specify the nature of the elementary mating act. One must make an assumption as to what goes into the mating process in the genetic sense and what kinds of particles and how many arise in it. Second, the model must specify the kinetic aspects of the mating pool, how frequently mating occurs, whether the matings are random or synchronized, and how the rate of extraction from the pool for maturation is related to the growth rate. Unfortunately, almost any theory of the elementary mating process can be made consistent with the experimental data if one adjusts the assumptions as to the kinetics in the pool with sufficient freedom.

Making the assumption that DNA is the sole carrier of genetic information in the infected cell limits the type of model that one can construct to a considerable extent, since an appreciable amount of data has been accumulated about the behavior of the phage DNA during the process of growth in the infected cell. We know from the work of Hershey (1953) that a pool of DNA begins to be formed shortly after infection and reaches a size of approximately 30 phage equivalents per cell at about the time of the first appearance of mature phage. After this time the pool size remains roughly constant until lysis takes place. The DNA in this pool behaves as precursor material to the DNA of the mature virus particles, and once it enters into the mature particles it does not return to the pool. The DNA in the pool has a duplication time of about 2 to 3 minutes, and in this time approximately 30 new mature phage particles are formed. If the cells are prevented from lysing, the growth and accumulation of mature phage particles inside the cells can continue for as much as 90 to 100 minutes, at which time over a thousand newly formed particles are, on the average, contained in each cell.

As a model of the genetic phenomena, we consider a pool of vegetative particles which can multiply and mate but which are not themselves infectious (Visconti and Delbrück, 1953). The process by which these vegetative phages are converted into complete phage particles will be called maturation, and particles will be thought of as being extracted from the mating pool for maturation. The mature phage particles become converted to the vegetative particles at the time of injection into a new bacterial host, and the maturation is irreversible within any cell. After the infection process starts, the vegetative phage contributed by the parental particles multiplies and the pool size of vegetative particles increases until it becomes stabilized by extraction for maturation. It reaches a stable level at which the rate of multiplication of particles in the pool is just equal to the rate at which particles are extracted from the pool. Mating takes place in this pool at a rate which for the phage T2 is approximately as high as that of the multiplication (Levinthal and Visconti, 1953), but in the phages T1 and  $\lambda$  is very much lower.

### *B. Distribution of Mutants*

If a mutation should occur in this pool of vegetative particles, the total number of mutant phage it would contribute to the final burst would depend on when during the latent period it occurred. Since the pool is constant in size, every particle, once it is formed, must have a probability of one-half of being extracted from the pool and an equal probability of undergoing further duplication. If it is duplicated, then each of the two daughters has a probability of one-half of being extracted and one-half of undergoing further growth to produce additional particles. Thus, except for the effect of mutations which occur just prior to lysis and before the pool reaches constant size, 50 % of them would lead to only a single mutant phage particle in the final mature progeny, and approximately one-eighth of the mutational events would yield the two daughter mutants in the burst. The distribution of mutants per mutational event can be calculated (Levinthal, 1957) by considering successively the probability of extracting daughters, granddaughters, etc., and the results can be represented to a good approximation as follows:

$$p(x) = \frac{.5}{x^{1.5}} \quad (1)$$

$p(x)$  is the probability of finding a clone of size  $x$  in the progeny of a single burst. Luria (1951) measured this distribution for the phage T2, and from the observed results he was able to conclude that a mutant which arose in a cell was itself able to multiply and produce a clone of mutants. The distribution he obtained was not significantly different from that predicted by equation (1) except that it had more very large clones than the calculated distribution.

This is not surprising in terms of the model discussed here since no account was taken of the mutations which occur very early, before the pool reaches constant size; these would be expected to produce large bursts. For the events which do occur during the period of the constant pool, the average number of mutants observed per mutational event is equal to the burst size divided by twice the number of particles in the pool. This can be seen by noting that whenever a mutation occurs in the pool, on the average  $1/N$  of all future progeny to mature will be mutants if  $N$  is the pool size. If the mutation is equally likely to occur at any time in the constant pool, then the mean number of particles produced after the event is just half of the burst size.

### *C. Distribution of Recombinants*

We would expect the calculations given here to be somewhat more reliable for recombinants which arise in an infected cell than for mutants since the probability of collision which could lead to a mating event would be expected to increase as some power of the number of particles in the pool. Thus, most of the mating events in a normal cross will occur after the pool has reached its constant size. We will define  $\epsilon$  as being equal to the average number of observed recombinants per observed recombination event; as in the case of the distribution of mutants, this will be equal to the burst size divided by twice the mean number of particles in the pool. The mean number of observable recombinational events can be determined only in those systems in which the recombination between two markers is rare. Under these conditions a significant fraction of the single bursts will show no recombinants of a particular type; from the fraction which shows none, one can calculate the mean number of events under the assumption that the events are distributed at random. For T1,  $\epsilon$  is found to be approximately 2.5 and the burst size is about 100 (Bresch, 1955). Thus, the mean number of particles in the pool is approximately 20. This calculation for the phage T2 leads to similar results (Hershey and Rotman, 1948) in rough agreement with the chemically measured size of the pool; but unfortunately the data used in the calculation are not as reliable as in the case of the phage T1 because of the large number of mating events, whereas in T1 the chemical analysis of the pool size has not yet been made. The few available experiments (Bresch, 1955) which give the distribution of the number of recombinants per recombination event for markers which are not extremely close do agree roughly with formula (1) but the data are not adequate for a critical test of this point.

### *D. The Mating Process*

The above considerations apply only to the kinetics of the pool of vegetative phage and do not require a specification as to the nature of the elementary mating event. In order to complete the model we will assume that the

elementary mating event involves the pairing of two particles, which cooperatively produce a third which contains the overlap region where the contributions from one parent end and that of the other parent begins, as in Fig. 4 (c). We will assume that this is the only way recombinants can arise, but that particles can also duplicate by themselves without mating. If a heterozygous particle duplicates without mating, it is assumed to segregate and produce its two daughter types within the same cell in which it was produced. We will also assume that the mating is random within the vegetative pool and that mixing is complete so that no particles are excluded from the mating process. We will further assume that if a mating event of the type described here takes place, the probability of the overlap and the average length of the overlap will be the same for the phages T2, T1, and  $\lambda$ , but that the difference between these systems will be in the frequency with which the mating occurs, as compared to the growth without mating. Granting these assumptions, the fraction of the total progeny which will be produced in a mating event can be estimated for T2 as about one-half. This estimate can be obtained in several ways, but none of them is very accurate. One is from the frequency of double heterozygosis for two distinctly linked markers, which Hershey found to be 6 % as compared to the 2 % which is the frequency for a single marker. By selecting a plaque that shows mottling, one is selecting a phage which arose in a mating event between two particles which differed with respect to the *r* marker involved. Thus, we are excluding from consideration those matings which are incestuous in that they involve pairing between genetically identical particles. At the first mating, two particles which differ with respect to the *r* marker must also differ with respect to all other markers, and one would expect the frequency of observed heterozygosity for an unlinked *h* marker to be double the usual value of 2 %. However, this effect would become less significant as more recombinants were formed in successive rounds of mating. If the probability of the occurrence of a heterozygous region were equal for all parts of the genetic structure and if every particle could be heterozygous, then we would expect to find about 3 % of the mottled plaques also heterozygous for a distantly linked *h* marker. The fact that one observes 6 % would by this model imply that about half of the observed particles in the progeny came from a mating event and the rest did not.

An analysis of similar recombinational data for T1 and  $\lambda$  indicates that there are far fewer mating events per growth cycle with these phages than with T2 or with T4. Therefore, it is very much less likely that any particle observed in the progeny will have arisen in a mating. This difference in the number of mating events also reflects itself in the number of heterozygotes observed in the progeny of the cross. Only those particles which have just arisen in a mating can have received genetic information from two different



parents, and growth after formation of the heterozygous particles is assumed to segregate it into pure types. The number of heterozygotes expected in the progeny can be calculated by considering that if such a particle is formed in a pool of constant size it has a probability of one-half of being extracted as such and a probability of one-half of being segregated as it duplicates. Thus, there will be one mature heterozygous particle which arises directly from a mating for every two mating events that occur in the pool. In T1, with a maximum of 13 % recombinants between distant markers, about 25 % of the particles in the output must have either mated or been the descendents of particles which mated nonincestuously. Each mating event produces on the average about 2.5 recombinant particles ( $\epsilon = 2.5$ ) and the burst size is about 100, so we can conclude that there must have been on the average  $100 \times .25/2.5 = 10$  recombinant producing events in each infected cell. But only in half of these events would the immediate product of the mating have been extracted by maturation without further division, so that only in five particles in a burst of one hundred would it be possible to find heterozygosis. In T2, when one selects for particles which came only from a mating by selecting those which are heterozygous for one marker, one finds 6 % are heterozygous for another marker. If the same frequency obtained in T1, one would expect 6 % of the five particles per burst to be heterozygous for any one marker. Thus one would expect to find of the order of three heterozygous particles per thousand progeny. This expectation is to be compared with about one per thousand found by Trautner (1957). The same type of analysis for the phage  $\lambda$  yields similar results. It is not yet clear whether this difference between the observed and the expected values is significant or is due to the various uncertainties in the measurements.

#### *E. Distribution of Recombinants for Close Markers*

There is one additional prediction which comes out of this model. If two markers are closer together than the length of the heterozygous overlap region, the distribution in the number of recombinants produced per recombination event is changed significantly. The exact nature of the new distribution will depend on whether the heterozygous particle which yields one parental type and one recombinant is scored as a recombinant or not. In an experiment reported by Stahl (1956), using two rII mutants, heterozygous particles of this type would have been scored as recombinants; in this case one expects a very different distribution in the number of recombinant particles. For now, instead of the heterozygous particle segregating to produce two recombinants, it segregates to produce one recombinant particle and one parental, so that the probability of getting a clone of size one, instead of being 50 % as in the case of distantly linked markers, becomes 75 % (50 % being the probability of extracting the heterozygote and an additional 25 %

for the probability of extracting the single segregant in the next generation). And the value of  $\epsilon_c$  for closely linked markers becomes:

$$\epsilon_c = \frac{\epsilon_d}{2} + \frac{1}{4}$$

where  $\epsilon_d$  for distant markers is as before, the burst size divided by twice  $N$ , the pool size. This calculation, which gives  $\epsilon_c = 1.50$  for T4, is in better agreement with the results obtained by Stahl (1956); however, in this case the markers used were very closely linked, and the results may be affected by the negative interference which occurs over very short distances of the genetic map.

## VII. FINE STRUCTURE GENETICS

### A. The *rII* System

In 1955 Benzer made a discovery which opened up a completely new class of investigations in phage genetics. This discovery was of a purely technical nature, but its ingenious exploitation made possible a kind of genetic analysis which had not previously been possible either with phage or with any other organism. Benzer found that a strain of *E. coli* K12, lysogenic<sup>1</sup> for the phage  $\lambda$ , would not produce plaques when used as an indicator bacterium for a certain class of *r* mutants of the phages T2 and T4, whereas other variants of these phages were able to plate on K12 ( $\lambda$ ) with an efficiency which was almost as high as on the usual indicator, *E. coli* B. Although this system shows a superficial similarity to the host-range mutants discussed earlier, it has many differences which make it very much more satisfactory for detailed genetic analysis; *r* mutants arise spontaneously with a frequency which is of the order of 1 in 10,000, and Benzer found that about two-thirds of these mutants fell into the *rII* class, that is, they did not plate on K12 ( $\lambda$ ). The isolation of a large number of *rII* mutants of independent origin is thus not excessively difficult. But the more important point is that when two of these mutants are crossed with each other, using B as the host cell of the cross, wild-type recombinants are produced as in any cross except that the recombination frequencies are all small. By plating the progeny of the cross on K12 ( $\lambda$ ), one can selectively count these recombinants, since only they or spontaneous reverse mutants will produce plaques on this indicator. In those cases where the number of recombinants is sufficiently large so that the comparison can be made accurately, the number of wild-type recombinants, as determined by direct observation on the cell B and by selection of plaques on K12 ( $\lambda$ ), is the same. This means that one has a simple and direct method

<sup>1</sup> See Chapter 9 for a definition and discussion of lysogeny. From the point of view of the present discussion, K12 ( $\lambda$ ) is simply another strain of *E. coli*.

of determining the number of recombinants in a cross even when that number is extremely small. Benzer has estimated that a recombinant frequency of  $10^{-8}$  (one in a hundred million) can be detected if the reverse mutation rate of the parents is sufficiently low.

More than two thousand rII mutants have been mapped with varying degrees of precision. The results of a series of two-factor crosses can be summarized by the construction of a one-dimensional map on which most of the mutants can be arranged as a set of points. The distances between these points are proportional to the recombination frequency between the mutants, and the distances are roughly additive. Therefore, the first conclusion from this work is that the general ideas discussed above regarding a linear genetic map apply even to these closely linked markers.

### *B. Deletions*

In addition to the mutations which behave as though they occupied a point on the genetic map, about 10 % of the rII mutants studied behave as though they occupied an extended region. These mutants, which are called "deletions," also differ from other rII's in that they show no evidence (less than one in  $10^8$  in a stock) of spontaneous reversion to wild type. In practice the deletions can be selected by testing all mutants for their reversion rate and selecting those which show none. The definition of a deletion which was used by Benzer is "a mutation which fails to give any wild recombinants with each of three different mutations, all of which do give recombination with each other." In order to apply this test, the necessary mutants must be mapped and tested; this has only been done for a small number of the non-reverters. However, in all cases where the nonreverters can be tested they are found to be true deletions. Thus, it is reasonable to assume that all the nonreverters represent deletions in the genetic map.

The availability of the deletions makes possible an essentially new and very elegant demonstration of the linearity of the genetic map. Analytical methods have been developed for treating the interactions of many deletions, the principle of which can be seen in Fig. 6. If the genetic map could be represented on some kind of a two-dimensional surface, there should occasionally be found two pairs of mutants such that each pair would give recombinants when the two members of the pair were crossed with each other; but no cross between members of different pairs would yield any. Such a result would require a cyclic representation as shown in Fig. 6 (a). This situation has never been detected, and the observed data are all consistent with a one-dimensional representation such that if two mutants recombine with each other and a third does not recombine with either of them, then the third must lie between the two in the sense that mutant B is between A and C of Fig. 6 (b). Since all mutually overlapping deletions can be unambiguously

ordered by this type of analysis, one can again conclude that the genetic map must be a one-dimensional structure. The special and important characteristic of this demonstration is that it does not in any way depend on a determination of recombination frequency, but only on the presence or absence of recombinants in crosses between pairs of mutants.

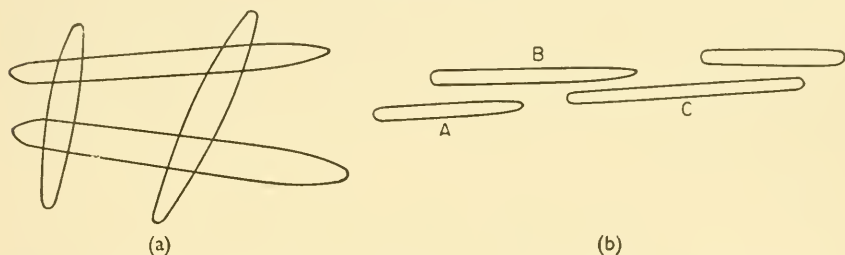


FIG. 6. Possible arrangements of deletions in a genetic map. If the map were two-dimensional, one should occasionally observe deletions which overlap as in (a). All of the observed data are consistent with a model of the form (b). Any overlapping deletions in either model mean that no recombinants are observed between the two mutants.

### C. Function Units

During the course of these investigations, Benzer also found that the rII mutants could be divided into two classes by a test which did not involve genetic recombination but that seems to be based on the function of various parts of the genetic material. If two different mutant phages are both allowed to adsorb to a single K12 ( $\lambda$ ) cell, one sometimes finds the cell yielding few or no progeny phage; with other pairs of mutants the cell yields an essentially normal burst. It is found that all point mutants of the rII class can be divided into two classes, *A* and *B*, such that any two members of the same class, if infecting a single cell, will not yield a normal burst, whereas any two mutants in different classes, infecting the same cell, will yield a normal burst. This method of dividing the mutants into the two classes obviously does not involve the production of recombinants, since some pairs of mutants which are in the same class are less closely linked than other pairs in which the two mutants are in different classes. However, it is found that this division into the two classes also corresponds to a separation on the genetic map. All of those mutants which are in class *A* lie at one end of the genetic map while all those in class *B* lie at the other end of the map. Benzer called these two regions "cistrons" by analogy with a *cis-trans* genetic test which is used in higher organisms.

The most direct interpretation of these findings appears to be that suggested by Benzer. In this picture a cistron is a region of the genetic map defined in terms of the test used to separate *A* mutants from *B* mutants. It



is thought of as being responsible for the production of some substance; let us say all or part of a protein molecule. In order to produce viable phage, the infected cell must produce both the *A* substance and the *B* substance intact. A cell infected by a mutant will, in general, not make a functioning substance; the protein molecule may not be made or it may be made in a sufficiently altered form so as to be nonfunctional. Therefore, when two different *A* mutants are added to a K12 ( $\lambda$ ) cell, the infected complex does not have a good copy of the *A* substance, whereas if an *A* mutant and a *B* mutant are added, one produces the *B* substance and the other the *A* substance, so that the infected complex has the material necessary to grow new phage.

Some of the mutants tested are capable of undergoing a certain amount of growth in the cell K12 ( $\lambda$ ). These mutants, which are designated as "leaky", would under this picture produce a defective, but not totally nonfunctional substance, while the nonleaky mutants and, presumably, the deletions would be ones which were unable to make a product which functioned at all.

#### *D. Negative Interference*

In many organisms a crossover occurring at one point on the genetic map reduces the probability that another crossover will occur in the same region of the map in the same mating event. In *Drosophila* (Weinstein, 1958), if very closely linked markers are used, a double crossover is much less probable than the product of the individual crossover probabilities. In phage crosses, on the other hand, there is an apparent *negative* interference which applies even to distantly linked markers. This means that in a three-factor cross  $abc \times a^+b^+c^+$  a *higher* frequency of recombination between the markers *b* and *c* is observed in that fraction of the progeny which is also recombinant between *a* and *b* than if all the progeny particles are examined. A double recombinant is thus *more* probable than would be predicted if the two recombinational events were independent. However, Visconti and Delbrück (1953) pointed out that this is an expected consequence of the multiple random matings. It is due to the selection of particles which have mated more frequently rather than one recombinational event affecting the probability of another. Since the number of rounds of mating in T2 is large and the estimate of it is rather uncertain, it is difficult to determine if any residual interference exists in this system. But with the phage  $\lambda$  the total number of rounds of mating is only about one-half, and Kaiser (1955) was able to show quite clearly that interference does not exist in this system—at least for markers which are not closely linked.

If very closely linked markers are studied, then it is found (Streisinger, 1956; Chase and Doermann, 1958) that a pronounced negative interference does exist. Using several closely linked rII mutants, Chase and Doermann

found that the double recombinants were twenty to thirty times as frequent as would be predicted if the recombinational events were independent. This same result has been obtained with very closely linked markers in other organisms, and a variety of explanations have been proposed. One proposal is that switching events to produce recombinants occur in clusters along the linkage map. These clusters are not ordinarily observed as such, since if the markers used are distant compared to their size, only those which contain an odd number of switches produce recombinants. The analysis of the data in terms of these clusters is not yet complete (Barricelli, 1958), but preliminary results indicate that the mean number of switches per cluster may have to be only slightly higher than one to account for the observed results.

### VIII. PHENOTYPIC VARIATIONS

#### *A. Phenotypic Mixing*

In general, when a single phage particle infects a cell, the new phage particles produced are, except for the very rare mutant, identical with the infecting particle. It is, in fact, this stability of the phage's characteristics which makes possible the study of its genetics. There are, however, three known exceptions to this general rule. Cells singly infected with heterozygous particles are effectively mixedly infected, since they yield two progeny types as well as a few more heterozygotes which may be newly formed.

The output of a phage cross can contain, in addition to the heterozygotes, a second type of confused particle called phenotypically mixed (Novick and Szilard, 1951). These are genetically pure, since the plaques they produce contain phage which is all of the same type. However, they do not show the same phenotypic character when they first arise in the cross that they show after they have grown through one cycle in a new bacterium. Phenotypic mixing does not occur for all genetic markers; it seems to be limited to those which affect the protein coat of the phage, for example, the *h* mutants, which control the ability of the coat protein to attach to the bacterial surface. The explanation of this phenomenon, which fits all the currently available data, is that the molecules which will form the protein coats are made by all the genetic structures which are in the infected cell, and there is a random assortment of these new molecules with the genetic material at the time of maturation. Thus, one can explain the finding (Streisinger, 1956) that the phenotypic character of a particle liberated in a cross is determined more by the majority type in the cross than by the genetic characteristic of the particle. If this explanation is correct, then the phenomenon of phenotypic mixing is relevant to the problem of how the genetic material acts in making protein, but is not relevant to the problem of how recombinants are made.

### B. Host-controlled Variation

The third exception to the rule that a phage always reproduces its own kind is the phenomenon of host-controlled variation. It is not known whether this phenomenon is directly connected with genetic processes or not. The phenomenon itself, however, is very striking and seems to occur in all the well-studied phage systems. The first clearly recognized examples were reported by Luria and Human (1952), Ralston and Krueger (1952), and Anderson and Felix (1952). In each of these cases the ability of the phage to grow on one or more host strains is altered by a single passage in a particular cell type. The remarkable characteristics of this alteration which distinguish it from the selection of a spontaneously occurring mutation are that it can occur in all the infected cells and that a normal burst of altered phage is produced from each cell. Even more important is the fact that only the last cell through which a phage was grown but not any of its previous history determines its host range. The host-induced modifications produce a non-heritable change in the ability of a phage to grow in some particular hosts. The phage P22, for example, which was studied by Garen and Zinder (1955), plates with the same efficiency on the cells *Salmonella typhimurium* and *S. gallinarum* if it has been grown on *S. typhimurium*. However, the phage which is liberated by *S. gallinarum* is modified and it plates with an efficiency of  $10^{-5}$  on *S. typhimurium* as compared to plating on *S. gallinarum*. The situation here is adaptive in the sense that the modification induced by *S. typhimurium* results in an *increased* ability of the phage to grow on that cell while at the same time it may reduce the ability of the phage to grow on some other cell.

Although most of the host-induced modifications are adaptive (Luria, 1953), the one first described by Luria and Human is not. The phage T2 gives a normal yield when grown in *E. coli* B/4<sub>0</sub>, which is a one-step mutation from *E. coli* B. However, the phage particles which are produced, designated T\*2, are unable to undergo further growth on either B or B/4<sub>0</sub> except for about  $10^{-3}$  of the infected cells, which do produce phage. A related cell, *Shigella dysenteriae*, will yield progeny with either T2 or T\*2. In addition, the fraction of cells which yield progeny when infected with modified phage depends very much on the growth conditions of the cells and on the medium in which the cells are infected.

In order to determine whether the host-induced modification is an alteration of the genetic material or a phenotypic phenomenon, it is necessary to know if the DNA of the modified phage is injected normally into the cell in which it will not produce progeny. The direct experiment to determine this with radioactive isotopes has not as yet been carried out because of various technical difficulties. Garen and Zinder (1955) showed that a modified phage which also contains a plaque-type mutation could contribute its genetic

marker to the unmodified phage which were produced in a mixed infection. They made a mixed infection of *S. typhimurium* cells, using particles which had been grown on *S. typhimurium* of one genetic type and particles of another genetic type which had been grown on *S. gallinarum*. About half of the cells liberated at least one phage which had the genetic marker introduced by the modified parent. However, the contribution to the burst of the genetic marker from the modified phage was very much less than that of the marker from the unmodified phage. Under these conditions of mixed infection one can conclude that many of the cells incorporated the genetic material of the modified phage. However, this is not proof of normal DNA injection by the modified phage, since it is possible that the addition of the unmodified particles to the same cell altered the membrane in such a way that some injection could now take place.

Luria and Smith (1958), in further studies of the T\*2 system, have shown that although the modified phage adsorbs normally to *E. coli* B, two-thirds of the particles adsorbed do not kill the bacterium. Nor does the modified phage with the  $r^+$  marker contribute to the growth of T2rII in K12 ( $\lambda$ ). These authors conclude that the most likely explanation of the phenomenon is that there is genetically normal DNA in a faulty injecting system. If so, the modification is another example of nongenetic, phenotypic variation.

## IX. CONCLUSION

Much of the work on phage genetics has as its aim the understanding of the elementary processes in terms of the molecular interactions occurring in the infected cells. Any genetic system must be such that it can be duplicated, that it can mutate, and that it can form genetic recombinants. In addition the genetic material must be able to control the formation of the various enzymes, structural proteins, and other components required for the development of the organism. During the last few years there have been great advances in understanding the first of these properties. The discovery by Avery and associates (1944) that DNA can transfer genetic information from one bacterium to another, and the work of Hershey and Chase (1952), which demonstrated that the phage DNA and protein are separated at the time of infection, show that in these organisms for at least a part of their life cycle DNA does contain the genetic information. The elucidation of the structure of this molecule by Watson and Crick and the attendant suggestions which it gave as to how this molecule could be duplicated have been followed by intensive isotopic studies aimed at testing the hypothesis which developed from the molecular model. All the experiments performed on phage (Levinthal, 1955, 1956), on *Vicia faba* (Taylor *et al.*, 1957), and most definitely on



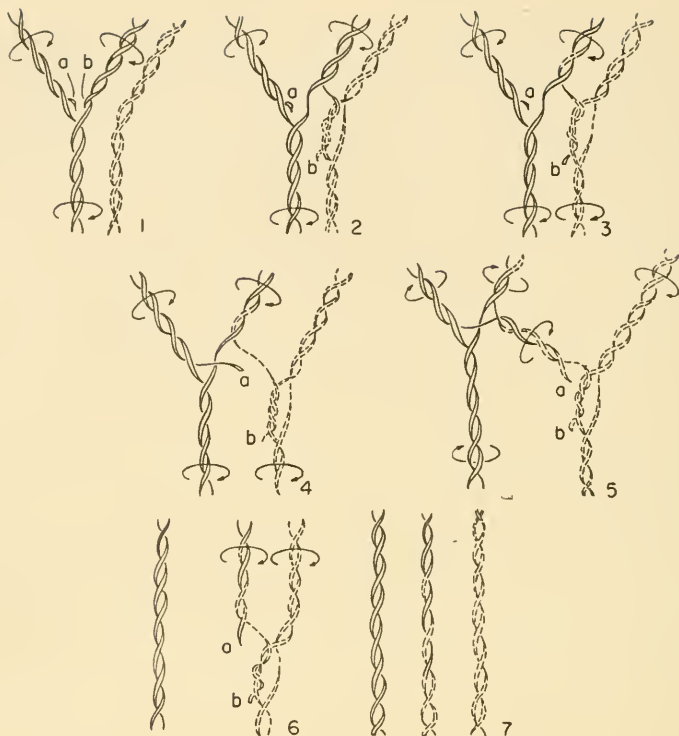


FIG. 7. A possible scheme by which heterozygotes could be formed by an interaction between two DNA molecules without breaking either of the parental molecules in the process. In (1) a particle which is in the process of duplicating pairs with another of different genetic composition. The pairing might be due to regions of the molecules in which the hydrogen bonds connecting the two chains are momentarily open. (2) One of the two growing strands switches and starts copying along a homologous region of the other parent. (3) Growth of the two new strands continues with each of them copying from a different parent. (4) After a period of continued growth and perhaps some separation of the two major chains, the second growing strand switches and starts copying along the *other newly formed* strand. (5) The two strands are pulled apart by thermal agitation accompanied by unwinding of the newly formed double helices and re-winding of the new one. This process requires no over-all energy expenditure and releases free energy due to the separation. (6) One of the parents is completely restored to its original state. The rest of the growth is completed, either as shown or with the second growing strand (a) copying along the free parental next to (b). Finally the process is finished (7), with the recombinant being made of completely new atoms and the parental structures unchanged.

A double or triple recombinant could occur in two ways. After state (4), when the second chain has been copying along the dashed parent for a short time, it might revert back and copy for a short additional period along the solid parent. It would still have to return and complete the process, as indicated, before separation of the two parental structures could occur. This would lead to multiple switches for one of the chains occurring over a short region of the map. However, this cluster of switches would always contain an *odd* number of switches. A multiple recombinant could also be formed if a second major switch occurred after stage 6.

bacteria (Meselson and Stahl, 1958) have lent support to the self-complementary mechanism of duplication originally proposed by Watson and Crick (1953).

It is possible (Levinthal, 1957) to construct a model of phage genetics which involves only simple modifications of this self-complementary scheme and which is consistent with the experimental results so far reported for the phage system. Unfortunately, however, one does not know enough of the physical chemistry of the DNA molecule to evaluate the plausibility of all the interactions that would be necessary in such a model. The difficulties are not connected with uncertainties of the regular structure given by the crystallographer but, rather, the difficulties concern the question of how the molecule behaves in the intracellular environment in which the recombination processes would be taking place. We can only estimate very crudely, for example, the fraction of the hydrogen bonds connecting the two chains of the double helix which will be opened at any one time. However, making what appear to be reasonable assumptions about the way in which DNA molecules would behave in such an environment, one can obtain a self-consistent scheme which does account for all the observations. The essentials of this scheme are shown in Fig. 7 and are presented primarily to demonstrate that there is no inherent difficulty in producing such a model. From the overall point of view this model results in the interaction of two DNA structures which together produce one new particle which has a heterozygous region at the point at which the copying changes from one of the parental structures to the other; in this region negative interference can occur in that at least one of the two chains can show several switches. In this scheme it would be assumed that particles can also duplicate without mating, that is, one particle could make two as well as two making three. The model also makes some rather specific predictions as well as accounting for currently available data. For example, it suggests that whenever negative interference occurs, that is, whenever there are several switches in the same small region, the total number of switches must always be odd; the mating which produced the particle of negative interference will produce a heterozygous particle with the overlap in the region in which the negative interference occurs and the multiple switches will occur in only one of the two strands of the heterozygous particle. These predictions can in principle be tested by purely genetic experiments.

If all of the implications of this model are taken seriously, it follows that high negative interference could occur during the local pair-wise interaction which leads to the production of heterozygotes. On the other hand, group mating would result if a second switch were to occur to a different parental structure after the first had been completed. Experiments of Edgar and Steinberg (1958) indicate that the interaction which produced high negative

interference is pair-wise and does not involve a third partner. However, Edgar (1958) has also obtained results which indicate that the negative interference occurs not when the heterozygous particles are being formed, but when they produce further progeny. He finds that the number of observed wild-type recombinants arising in a cross between very closely linked rII markers increase by a large factor if the progeny of the cross is allowed to grow for one cycle in B cells before being plated on K12 ( $\lambda$ ). The only apparent source of this increase is the heterozygous particles in which the overlap spans both markers. This point can be definitively established, however, only by single-burst experiments in which the progeny of individual heterozygotes are examined.

It is certainly not yet possible to establish in any detailed sense a molecular model of the recombination process in phage. Since, however, this is the primary goal of much of the work on phage genetics, further studies will probably combine chemical and physical studies with the more formal aspects of genetic analysis.

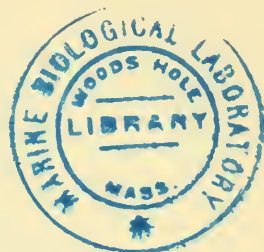
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## Chapter IX

### Lysogeny

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#### I. INTRODUCTION

The clearest manifestation of bacteriophage action and, consequently, the one which was first observed and thereafter received the greatest attention,

is brought about by bacteriophages that rapidly and completely lyse cultures of susceptible bacteria: infection with such phages leads irrevocably to death and dissolution of the host.

It was soon recognized, however, that another type of relationship could exist between bacteria and bacteriophages: this type is represented by lysogenic bacteria, which apparently can be grown indefinitely while releasing phage into their culture medium.

These two situations therefore appear to be completely opposed: in the first case, bacteriophage is the agent of an acute and always fatal disease of the host, while, in the second case, it behaves as a perfectly well tolerated parasite. For many years, the nature and mode of action of bacteriophage were subjects of controversy; conflicting theories were proposed, depending on which facts, whether belonging to one or the other situation, had to be accounted for. It is only in the last decade, after well-defined cases have been thoroughly analyzed, that a complete and harmonious picture has been reached.

Those bacteriophages which always kill the infected bacteria are now called *virulent* phages, and their properties and reproduction have been the subject of previous chapters in this volume. As to the phages which are produced by lysogenic bacteria, infection of sensitive *indicator* bacteria with such phages results either in multiplication of the phage and lysis of the infected cell, or it may lead to the formation of a stable association between the host and the virus. The infected bacterium survives and becomes hereditarily endowed with the property of producing new phage particles identical with those that have been used for the initial infection. A new lysogenic system has thus been established. Phages which are able to perform this process of lysogenization are called *temperate* phages.

Lysogeny may thus be defined as the hereditary property of certain bacteria which enables them to produce bacteriophage in the absence of external phage particles. A lysogenic bacterium possesses and transmits to its progeny the capacity to produce phage. Lysogeny is a stable character and, in a lysogenic culture, each bacterium will give rise to a lysogenic clone (Fig. 1).

"Artificial" lysogenic strains, that is, the lysogenic derivatives obtained by lysogenization of sensitive bacteria by temperate phages, are, in all their properties, similar to the lysogenic strains isolated from natural sources. A lysogenic strain is thus represented by the symbol of the bacterial strain, followed by the symbol of the phage in parenthesis. For example, a strain of *Escherichia coli*, called K12, which is lysogenic for a temperate phage called  $\lambda$ , is represented as K12( $\lambda$ ). The fact that infection of a sensitive bacterium with a temperate phage may lead to the formation of a stable lysogenic system shows that the genetic information introduced by the infecting particle is thereafter indefinitely perpetuated in every descendant of the

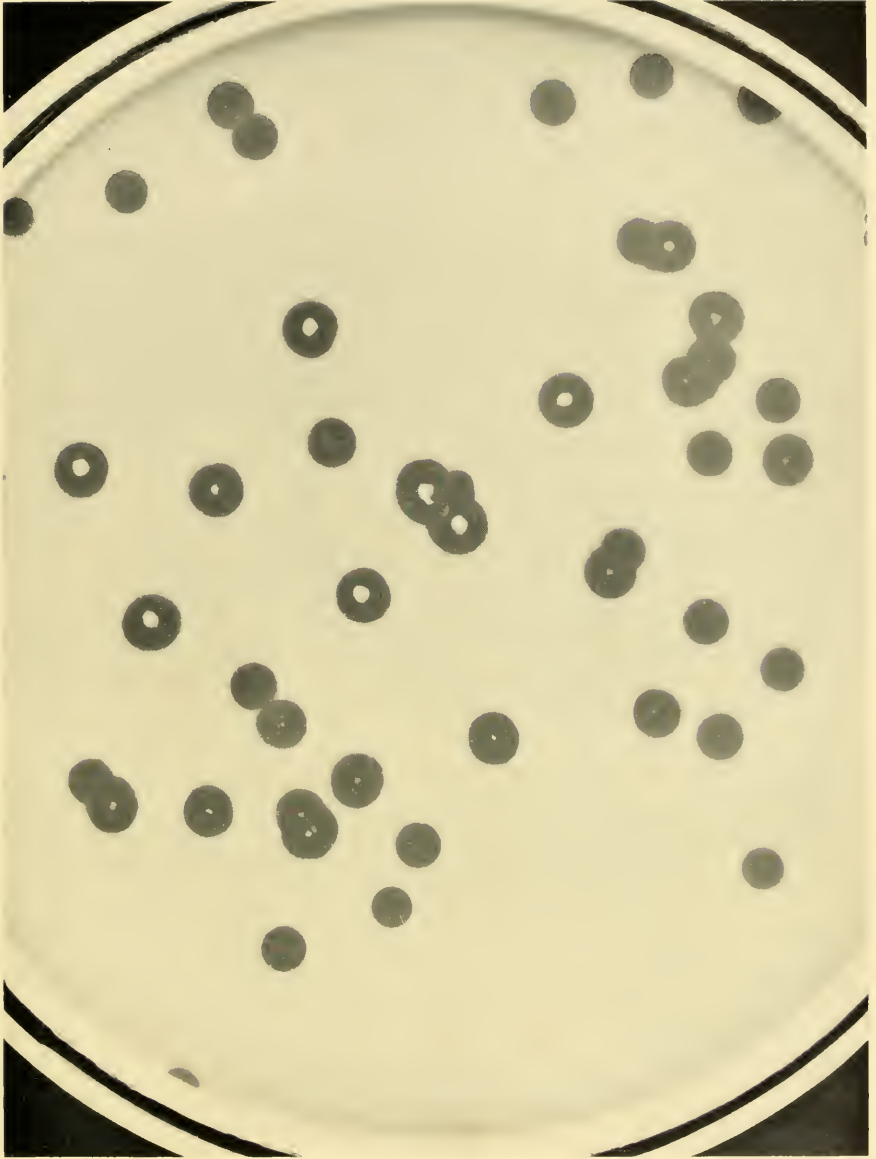


FIG. 1. Plaques formed on sensitive cells by plating samples of cultures of lysogenic *Bacillus megatherium*. Clear plaques are formed by free phages. Centred plaques are formed by colonies that liberate phage during growth. (Courtesy of Dr. H. Ionesco.)





original cell. It is, however, perpetuated, not in a form similar to that of the infecting particle, but in a noninfectious form, the *prophage*, which is integrated with the genetic apparatus of the host cell.

## II. HISTORICAL DEVELOPMENT OF LYSOGENY

### A. *Discovery and Criteria of Lysogeny*

Soon after the discovery of bacteriophage by Twort and d'Hérelle, it was observed by several workers, in particular by Bordet and Ciuca (1921) and by Gildemeister (1921), that strains could be isolated from natural sources which would produce bacteriophages active on bacteria of other strains. It was later realized that such phage-producing strains could correspond to one or the other of two different situations. In one case, the equilibrium between bacteria and phage is at the *population* level. In such *carrier strains*, phage production can be ultimately ascribed to a population equilibrium between resistant and susceptible cells, the latter being constantly reinfected by external phage particles. Bacteriophage-free cultures can be easily obtained by serial colony isolations or by treatments which will inactivate free phage or prevent its adsorption onto the host cells.

Completely different is the case of the *lysogenic strains*, in which the association between the phage and the bacterium is at the *intrabacterial* level. As shown by Bail (1925) and by Bordet (1925), after numerous isolations of single colonies, each colony will still give rise to a phage-containing culture. When lysogenic bacteria are grown under conditions which prevent reinfection with external phage particles, as for example, in a medium containing specific antiphage serum (MacKinley, 1925), each bacterium is able to give rise to a lysogenic clone. It was thus clear that the ability of lysogenic bacteria to produce phage is independent of the presence of free phage in the surrounding medium and is a property of each single cell of the culture.

Another important finding of Bail (1925) and of Bordet (1925) was that when sensitive bacteria are exposed to the phage produced by lysogenic strains, some of the bacteria survive and give rise to lysogenic clones. Moreover, the phage produced by such "artificial" lysogenic strains is in all respects identical with the phage used for the initial infection.

### B. *Concept of Prophage*

Each bacterium of a lysogenic strain, whether isolated from nature or "artificially" prepared by lysogenization, is therefore able to perpetuate the ability to produce phage. This ability appears, as written by Bordet in 1925, to be "inscribed in the heredity texture" of the bacterium. But under what

form is the ability to produce phage perpetuated in lysogenic bacteria? It was shown by den Dooren de Jong (1931) that treatments which would inactivate free phage of lysogenic *Bacillus megatherium*, such as heating at 100°C. for 20 minutes, would leave unimpaired the lysogenic character of cultures obtained from spores which had been submitted to the same treatment. Moreover, when lysogenic bacteria are disrupted by various means, such as lysis with unrelated phages (Burnet and McKie, 1929) or lysozyme (Wollman and Wollman, 1936; Gratia, 1936), no infectious particles can be found. Phage, therefore, is not perpetuated in lysogenic bacteria in the form of infectious particles, but in a noninfectious form.

An important question raised by the existence of lysogenic bacteria is that of the mechanism by which infectious phage particles are produced from the noninfectious "anlage" (Burnet and McKie, 1929) that they perpetuate.

It is generally observed that in growing cultures of lysogenic bacteria, the bacteriophage titer runs parallel with the number of bacteria of the culture. Phage production could result equally from a continuous secretion in the course of bacterial growth or from the discontinuous lysis of a small but constant fraction of the bacterial population. The first hypothesis was generally favored—in particular, by Northrop (1939), who, by comparing the kinetics of the production of enzymes and phage, concluded that phage, like enzymes, was secreted by lysogenic bacteria in the course of their multiplication.

After an eclipse period of about ten years, these problems were reinvestigated by Lwoff. Multiplication of lysogenic *B. megatherium* and the appearance of bacteriophage were followed, not in mass cultures, but under the microscope, in pedigrees of bacteria isolated with a micromanipulator. It was thus observed by Lwoff and Gutmann (1950) that lysogenic *B. megatherium* may undergo up to 19 divisions without any release of phage into the surrounding medium. Thus, multiplication of lysogenic bacteria can proceed without phage production. In other clones, however, by its sudden disappearance, the lysis of a cell could occasionally be observed. Then, and then only, the presence of phage (about a hundred particles per lysed cell) was detected in the microdrop. Therefore, the production of phage by lysogenic bacteria is a discontinuous process; it is the consequence of the lysis of a small fraction of the lysogenic population. In the case of lysogenic bacteria, as in the case of sensitive bacteria infected with phage, the production of infectious particles is a lethal process, each producing cell liberating a full burst of infectious particles. Lysogenic bacteria can survive and grow only when they do not produce phage. Their capacity of producing phage is, and can only be, a potential character. To the noninfectious form of bacteriophage which is the bearer of this potentiality and which can develop eventually into phage, Lwoff and Gutmann have given the name of *prophage*. The

prophage may be visualized as a specific structure whose replication is coordinated with the division of the host bacterium, which it provides with the information necessary for the synthesis of phage particles.

As already stated, the lysogenic character of a strain is generally as stable as any other genetic character. Spontaneous loss of this character has, however, been observed in exceptional cases, such as the classic strain of *B. megatherium* (den Dooren de Jong, 1931). Accumulation of nonlysogenic variants can be favored by the prolonged growth of this strain under conditions which prevent the readsorption of free phages (Clarke, 1952). Isolation of nonlysogenic derivatives has also been reported in other cases, as, for instance, in that of several strains of *Salmonella typhi* (Anderson, 1951). The frequency of such occurrence may be, to a certain degree, increased by exposure of a lysogenic population to heavy doses of ultraviolet light (Lederberg, 1951).

As it will be seen later (Section VII, B), the prophage appears to be the genetic material of the phage located at a specific site of the bacterial chromosome. Loss of lysogeny would therefore correspond to the loss or inactivation of this specific structure. Lysogenization, on the contrary, corresponds to the acquisition of this new hereditary determinant. In a lysogenic bacterium, host and virus genomes are integrated and replicated as a single unit during the course of bacterial multiplication. The presence of the prophage apparently does not affect growth or multiplication of the host bacterium: the prophage behaves as a normal cell constituent. Only when the prophage-bacterium relationship is disturbed do lysogenic bacteria produce phage (see reviews by Lwoff, 1953; Jacob, 1954).

### III. DETECTION AND OCCURRENCE OF LYSOGENY

The lysogenic character of a bacterial strain can be recognized only when there is available a sensitive indicator strain on which the produced phage can multiply and form plaques. When investigating whether a given bacterial strain is or is not lysogenic, cultures of this strain must therefore be assayed by usual phage techniques, on a variety of other strains of the same or of closely related species. When plaques are observed on one or several of these strains, and are constantly formed by cultures grown from serial isolations of single colonies, the strain under test may be considered as being lysogenic.

When systematic investigations of this kind have been carried out, a large proportion of the bacterial strains examined, whether recently isolated from natural sources or taken from laboratory collection, were found to be lysogenic. In a careful study of 34 strains of *Salmonella enteritidis*, Burnet (1932, 1934) was able to recognize lysogeny in 27 strains. From these 27 lysogenic



strains, three antigenically different types of phage, A, B, and D, were isolated. Phage A was also frequently isolated from lysogenic strains of *S. paratyphi* A, *S. paratyphi* B and *S. paratyphimurium*, whereas phages B and D, as well as another, phage N, were found to be more rarely produced by strains belonging to these species.

Such findings have been made in a large number of bacteria species (sporulated soil bacteria, *Enterobacteriaceae*, *Pseudomonas*, *Vibrio*, *Corynebacterium*, *Staphylococcus*, etc.). Certain strains of lysogenic bacteria are able to release two or more different types of phages. Among the strains of *S. enteritidis* studied by Burnet, two yielded both phages A and B, and one phage A and D. Some lysogenic strains of staphylococci may produce as many as five different types of phages (Williams-Smith, 1948; Rountree, 1949). Lysogeny, therefore, appears to be widely distributed among bacterial species. Moreover, it must be pointed out that the non lysogenic character of any given strain can never be asserted. It is impossible to know whether such a strain, which did not give plaques on the indicator strains employed, would not have proved to be lysogenic if a suitable indicator strain had been available.

#### IV. PHAGE PRODUCTION BY LYSOGENIC BACTERIA

##### *A. Spontaneous Production*

Cultures of lysogenic bacteria in liquid medium generally contain particles of free phage. In a population of lysogenic cells, the ratio "free phage : bacteria" remains approximately constant during growth of the culture (Fig. 2). As shown by Lwoff and Gutmann, with single cells, phages are produced by the lysis of a small fraction of the population. The rarity and the discontinuity of phage production in a lysogenic population may also be demonstrated by a modified single-burst experiment, which allows an analysis of the production of phage by a large number of small populations during a short time of growth (Bertani, 1951; Jacob, 1952c). The bacteria which produce phage during the period of observation appear to be randomly distributed among the populations sampled, which indicates that the factors which determine phage production appear randomly in rare individuals.

The constant ratio "free phage : bacteria" in a growing population expresses the fact that, per generation time, a constant fraction of the population lyses and releases a burst of phage. The ratio is a function of the burst size and of the frequency of lysis, which can be expressed as the probability per bacterium, per generation time, to produce phage. This rate of spontaneous production can be accurately measured by single-burst experiments. For a given lysogenic strain grown in given conditions, the rate of spontaneous

production is a constant of the strain. It varies widely from strain to strain ( $10^{-2}$  to  $10^{-5}$ ), depending upon the type of prophage propagated. Thus, in polylysogenic strains, that is, strains in which every bacterium is able to produce two or more types of phages, each type of phage is generally produced at its characteristic rate, irrespective of the presence of the other prophages. When the spontaneous production of such strains is analyzed as described

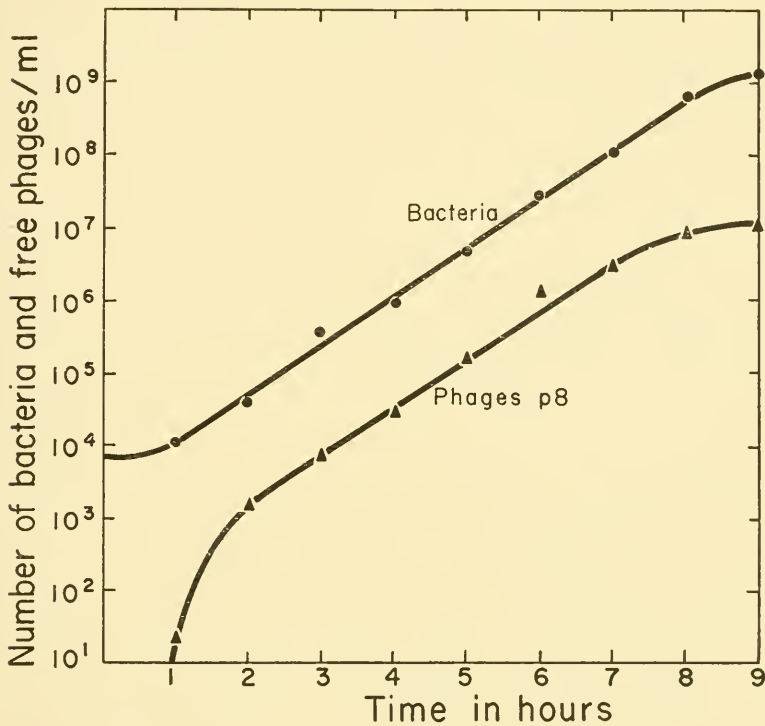


FIG. 2. Production of bacteriophages during the growth of lysogenic *Pseudomonas aeruginosa* 13 (8). The number of bacteria and of free phages/ml. are plotted on a logarithmic scale versus the time in hours. (From Jacob, 1954.)

previously, it is observed that, when the phages produced are unrelated, the spontaneous production of one type appears to be independent of the production of the other type (Bertani, 1951; Jacob, 1952c). For strains doubly lysogenic for certain types of related phages (Jacob, 1952c), however, both types are often released by the same lysing bacterium.

Thus, in a culture of lysogenic bacteria, the development of the prophage into phage occurs "spontaneously" in a small but constant fraction of the growing cells. The mechanism of this spontaneous production is still obscure.

However, whereas in some strains this spontaneous rate of production cannot be increased by artificial means, in other strains the production of phage can be induced at will in the whole population.

### *B. Induction of Phage Production in Lysogenic Bacteria*

In 1950, it was found by Lwoff and associates that when cultures of certain lysogenic strains of *B. megatherium* are exposed to suitable doses of ultraviolet light, the whole population lyses and releases phage particles. After exposure to UV light, bacterial growth first proceeds without divisions, during a time period corresponding to one or two generations. No phage is released during this period. At the end of this latent period, lysis begins and the first phage particles appear in the medium. If the proper dose of UV light has been used, lysis is rapidly completed and an average of about one hundred phage particles per lysed cell is released. During the latent period lysogenic bacteria can be prematurely disrupted in the same way as infected bacteria (with KCN + phage, lysozyme, chloroform, etc.). During the first two-thirds of the latent period, no infectious particles are found, but at the end of this time mature particles begin to appear and their number increases linearly with time until the full yield is completed.

Induction allows a careful analysis of phage production by lysogenic bacteria. Especially it makes it possible to compare phage development in the same phage-bacterium system, whether after infection of sensitive bacteria, or after induction of lysogenic bacteria (Figs. 3 and 4). It has thus been observed that the characteristics of phage development, such as latent period, average burst size, are very similar in both systems. Such an analogy indicates that the processes concerned with the actual multiplication of phage material, i.e. vegetative phase and maturation, are probably identical in induced lysogenic and in infected sensitive bacteria. For phage to be produced by lysogenic bacteria, there must be a shift from the stable prophage state to the transitory vegetative state. Once the vegetative state is reached, phage multiplication proceeds in the same fashion after induction of lysogenic bacteria as after infection of sensitive cells with homologous phage particles.

### *C. Factors Controlling Induction*

In the induced production of phage by lysogenic bacteria, it is convenient to distinguish three main factors: a genetic factor responsible for the susceptibility of lysogenic strains to the action of inducing agents; the inducing agent itself; and the physiological conditions which will allow the lysogenic cells to respond to the action of the inducing agent by the production of phage. These three factors will now be examined in turn.

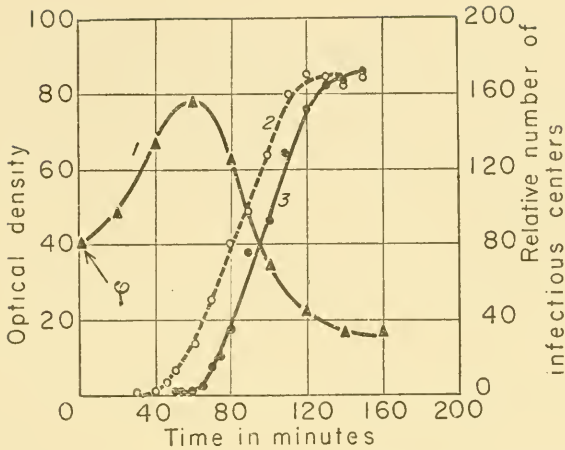


FIG. 3. Phage multiplication after infection of sensitive *Pseudomonas aeruginosa* 13 with temperate phage P8. Bacteria are infected with a multiplicity of 5 phage particles per cell. The optical density (Curve 1) and the relative number of infectious centers, measured after premature lysis (Curve 2), and during spontaneous lysis (Curve 3), are plotted versus the time of incubation at 37°C. after infection. (From Jacob and Wollman 1953.)

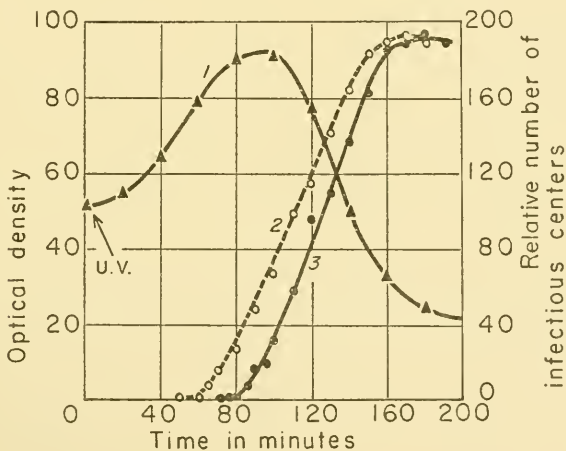


FIG. 4. Phage multiplication after U.V. induction of lysogenic *Pseudomonas aeruginosa* 13 (8). Bacteria are exposed to an optimal dose of UV light. The optical density (Curve 1) and the relative number of infectious centers, measured after premature lysis (Curve 2) and during spontaneous lysis (Curve 3) are plotted versus the time of incubation at 37°C. after irradiation. (From Jacob and Wollman, 1953.)



### 1. *Genetic Factor*

Exposure to UV light may induce phage development only in some lysogenic systems. Within the same bacterial species, both *inducible* and *non-inducible* strains can be isolated (Ionesco, 1951; Jacob, 1952c). Since a lysogenic system may be visualized in terms of an association between the bacterial host and the prophage, the question arises of whether one or the other component or the whole system is responsible for the character of inducibility. The answer to this question can be obtained by isolating lysogenic clones after infection of various sensitive strains of bacteria with various strains of temperate phages. In most of the systems which have been investigated so far, inducibility behaves as a characteristic of the *prophage*, although an exception to this rule has recently been reported (Ionesco, 1956). In fact, as will be seen later, the inducible character of a prophage seems to depend upon its location on the genetic apparatus of the host. In most of the lysogenic systems, inducibility and rate of spontaneous production appear to be correlated, the spontaneous production of inducible strains being higher than that of noninducible strains.

### 2. *Inducing Agents*

Inducing properties have been recognized for a number of agents. Physical agents, such as UV light (Lwoff *et al.*, 1950), X-rays (Latarjet, 1951), as well as  $\gamma$ -rays (Marcovich, 1956a), are very efficient. Among the chemicals that have been shown to act as inducers are nitrogen mustard (Jacob, 1952d; Williams-Smith, 1953), organic peroxides, epoxides, and ethyleneimines (Lwoff and Jacob, 1952), and azaserine (Gots *et al.*, 1955). Hydrogen peroxide also induces phage formation in certain inducible strains, either when directly added (Lwoff and Jacob, 1952), or when produced in a nascent state by addition of sulfhydryl compounds in the presence of copper (Lwoff and Siminovitch, 1952). Leucovorin previously irradiated with UV light has also been reported to have some inducing action (Borek and Rockenbach, 1955).

Most of these inducing agents also exert, in other systems, either mutagenic or carcinogenic activities. However, certain chemicals whose activities as mutagens or carcinogens have been demonstrated, do not induce phage production in those systems in which they have been assayed. This is the case with ethyl-urethane, caffeine, and also several water-insoluble carcinogens, such as methylcholanthrene and dibenzanthracene, the latter probably because they cannot penetrate inside bacteria. For those mutagens or carcinogens which may act as inducing agents, lysogenic bacteria provide a very sensitive test, since the probability of inducing phage development is in most cases very close to one.

Physical agents, such as UV light or X-rays, are particularly suitable for determining the dose-effect curves of induction. As shown in Fig. 5, the fraction of induced bacteria first increases with the dose of radiation. It reaches a maximum which varies according to the system under investigation and may be greater than 95 %. Then it decreases at a rate generally controlled by the bacterial "capacity" to reproduce phage, that is, by the

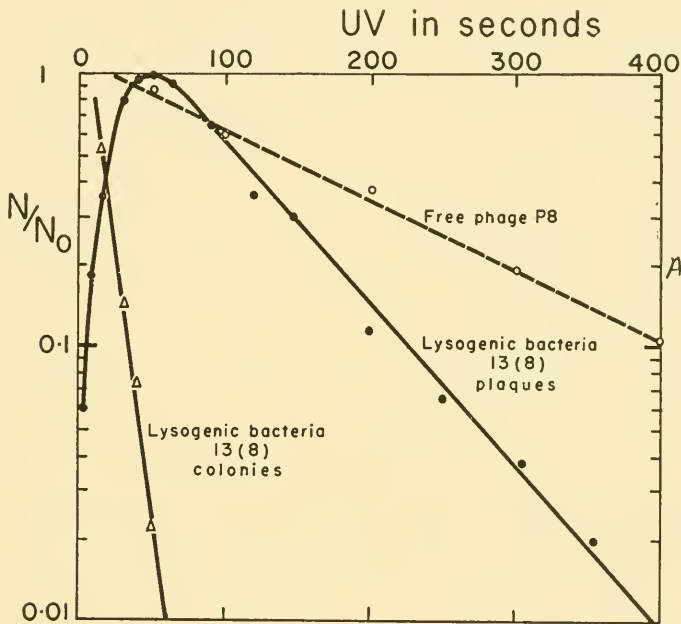


FIG. 5. Induction curve of lysogenic *Pseudomonas aeruginosa* 13 (8). The fractions of bacteria as colony formers and plaque formers, and of free phages P8 as plaque formers are plotted on a logarithmic scale versus the dose of UV light in seconds. (From Jacob and Wollman, 1953.)

survival, as a function of dose, of the bacterial ability to support multiplication of the phage under study (Jacob, 1954; Marcovich, 1956a). Because of their extreme sensitivity to the action of inducing agents, such as ionizing radiations, lysogenic bacteria may be used as a convenient material for the study of the biological effects of small doses of these agents (Marcovich, 1956b).

### 3. Environmental Factors

In order that exposure of inducible lysogenic bacteria to inducing agents lead to phage production, bacteria must be grown under suitable conditions.

Before exposure to radiations, the physiological state of the bacterial

culture controls *aptitude*, that is, the ability for bacteria to respond to irradiation by phage production (Lwoff, 1951). Disturbances in bacterial metabolism may affect aptitude markedly, as shown by the effects of carbon or nitrogen starvation before irradiation. In starved populations, both the proportion of bacteria able to produce phage and the sensitivity to UV light of these bacteria are strongly reduced (Jacob, 1952a). Amino acid starvation of amino acid-deficient strains produces the same effect (Borek, 1952). Thus, aptitude does not behave as an absolute property of the system, but undergoes variations controlled by the nutritional conditions of the system.

After exposure to radiations, environmental factors may still affect the *bacterial response* (Lwoff, 1951). Changes in the composition of the medium, or in the cationic balance (Huybers, 1953) after irradiation may thus prevent the production of phage in certain systems.

Like many other effects of UV irradiation, induction may be reversed by exposing irradiated bacteria to visible light (Jacob and Wollman, 1953). Photorestitution of the effects of induction does not take place after exposure to such agents as X-rays or nitrogen mustard.

Reversion of the effects of irradiation with UV light is only effective during the first quarter of the latent period. The bacteria thus recovered as colony formers still remain lysogenic.

#### *D. Mechanism of Induction*

Little is known about the mechanism by which phage development is initiated after exposure of lysogenic bacteria to an inducing agent. The question may be raised as to whether the primary effect is on the bacterial or on the prophage component of the lysogenic system. Two different kinds of evidence support the former hypothesis. One comes from the estimation of the size of the induction target by means of X-rays: induction appears to result from a single ionization and the size of the target found is too large to be the prophage itself. It appears to be analogous to the size of the whole bacterial nuclear system (Marcovich, 1956a). The other evidence comes from the analysis of spontaneous and induced phage production by polylysogenic bacteria carrying two different, but related inducible prophages: the correlation found in the productions of both types of phages (Jacob, 1952c) is incompatible with the hypothesis of a change of the prophage itself as the primary event of phage production. Development of the prophage appears therefore as a secondary effect, the primary event consisting of some alteration of the bacterium. The very nature of the inducing agents suggests that it could be an alteration in the nucleic acid economy of the host. It appears that in lysogenic bacteria the potentialities of a prophage cannot be expressed as long as the bacterial nuclear apparatus maintains control of the cell. Only when some disturbance occurs, either spontaneously or after the action of

inducing agents, can the prophage escape the control of the nucleus and develop into vegetative particles.

## V. PROPERTIES OF LYSOGENIC BACTERIA

In most cases, the lysogenic strains obtained by lysogenization possess the same properties as the nonlysogenic original strains, except for the specific properties which express the presence of the prophage. First of all is the ability to produce phage which, for any individual of a lysogenic population, is a potential character since its expression is lethal. Another property which is conferred on lysogenic bacteria by the presence of a prophage is their resistance to an infection by the phage they are able to produce or homologous phage. This property, called *immunity*, is expressed in every cell of a lysogenic culture.

Capacity to produce phage and immunity against the homologous phage or its mutants are in most systems the only recognizable differences between lysogenic and nonlysogenic derivatives of the same strain. However, in certain cases, other differences may be observed. The presence of a prophage may interfere with the ability of lysogenic bacteria to reproduce unrelated phages. It may even determine in the host some specific modification which, at first sight, would appear to be unrelated to lysogeny. These modifications, which are named *lysogenic conversions*, may bear on properties as different as the synthesis of a toxin or the formation of a surface antigen. Only by chance has it been possible to relate such alterations in bacterial physiology to the presence of a given prophage. These different expressions of the presence of a prophage will now be considered in turn.

### *A. Infection of Lysogenic Bacteria with Phages Homologous or Closely Related to the Prophage : Immunity and Incompatibility*

Lysogenic bacteria, as already observed by Bail (1925), Bordet (1925), Burnet and Lush (1936), Wollman and Wollman (1936), are not susceptible to the action of the phage they release, although they generally adsorb it. When infected with homologous particles, no apparent alteration of growth is observed, and the infecting particles do not multiply. This property of lysogenic bacteria is called *immunity*. Immunity of lysogenic bacteria is a form of resistance completely different from inability to adsorb, as exemplified by B/2 unable to adsorb T2. A lysogenic bacterium is always immune against the homologous phage or mutants of this phage. It may or may not be able to adsorb this phage according to whether or not it possesses the homologous receptor. For instance both *E. coli* K12( $\lambda$ ) and K12/ $\lambda$  are resistant to phage  $\lambda$ , the former because it is immune, the latter because it is unable to adsorb it. From the lysogenic derivative K12( $\lambda$ ) which adsorbs the



phage but is immune to its action, a mutant K12( $\lambda$ )/ $\lambda$  may eventually be obtained which will not adsorb phage  $\lambda$ .

Immunity is the expression of the presence, in the bacterium, of a prophage homologous to the infecting phage. When the prophage, either spontaneously or after induction, enters the vegetative state, immunity disappears and the superinfecting phage starts multiplying. This can be easily demonstrated when lysogenic bacteria are superinfected with a mutant of the homologous type (Bertani, 1953a; Jacob and Wollman, 1953). When, for instance, inducible lysogenic bacteria are first induced and then infected with an adequate multiplicity of a mutant of the homologous phage, each bacterium releases particles of the prophage as well as of the mutant type.

What is the fate of the phage material which has thus penetrated lysogenic bacteria in their normal noninduced state? In the vast majority of the infected cells, it does not either multiply or lysogenize: it is diluted out at each cell division, as can be accurately measured in inducible systems (Jacob, 1954). This material must thus be in a different state from either the prophage or the vegetative phage, since it is neither replicated as the former nor does it multiply as the latter. Its probability of developing into vegetative particles is very small, since this event can only occur if the prophage itself also develops. Its probability of becoming a prophage is also very limited, as shown by Bertani (1953a). Whereas lysogenic bacteria can easily be lysogenized by temperate phages unrelated to the prophage type, lysogenization with a phage homologous to the prophage is a rare event: there is *incompatibility*, at the prophage level, between related phages. In some cases, the infecting type replaces the original prophage type. In rarer cases, the infecting type is replicated together with the original prophage type. In such double lysogenic bacteria, where the two prophages differ by two or more genetic characters, genetic recombination in the prophage state has been observed (Bertani, 1953b; Appleyard, 1954b).

The presence of a given prophage therefore prevents both multiplication and lysogenization by a homologous infecting phage. Immunity reflects a block in the processes by which the infecting material of a phage enters the vegetative state, but the nature of this block is as yet unknown. Incompatibility, as will be seen in Section VIII, is an expression of the specific binding of the existing prophage to a chromosomal site of the bacterium.

### *B. Infection with Unrelated Phages*

Generally, a prophage does not interfere with the reproduction of unrelated phages, either virulent or temperate. For example, *E. coli* K12( $\lambda$ ), lysogenic for  $\lambda$ , exhibits the same sensitivity as nonlysogenic *E. coli* K12 to infection with virulent phages T1 or T5. In the same way, bacterial responses to infection with unrelated temperate phages are generally not modified by

the presence of a prophage. For example, if lysogenic *E. coli* K12( $\lambda$ ) is infected with phage P1, responses to infections are similar to those found with nonlysogenic *E. coli* K12: a certain fraction of the bacteria lyses and produces phage, whereas another fraction survives and becomes lysogenic. The latter clones are then doubly lysogenic and carry both prophages  $\lambda$  and P1.

However, it is sometimes observed that a lysogenic cell does not react to phage infection in the same way as a nonlysogenic cell of the same strain. In such cases, the lysogenic bacterium is often unable to ensure the multiplication of a phage which is able to develop on the nonlysogenic one. This is the case with many strains of *Salmonella typhi* in which the presence of a variety of prophages (type-determining phages) prohibits multiplication of a phage active on the nonlysogenic derivative (Anderson and Felix, 1953). Similarly, strain *Sh* of *Shigella dysenteriae* is sensitive to infection with the phages of the T series. Lysogenic *Sh*(P2) fails to support the complete development of T2, T4, T5, and T6. But the infected bacteria are killed without lysis or phage production (Bertani, 1953a). Very remarkable is the observation by Benzer (1955) that the presence of prophage  $\lambda$  in *E. coli* K12( $\lambda$ ) prevents the multiplication of  $r_{II}$  mutants of T2, T4, and T6, but not of any other mutants of the same phages. As for immunity, this phenomenon reflects a specific block in the multiplication of the infecting particles.

Besides preventing phage multiplication, the presence of a prophage may also induce phenotypic modifications of unrelated phages which multiply in lysogenic bacteria. An example of such modifications is the "adaptation" of the *Vi* phages of *Salmonella* (Craigie and Felix, 1947) analyzed by Anderson and Felix (1953). *Vi* phages II have a common host called A. When the original phage type is plated on different *Vi* positive strains, it forms only a small number of plaques. From the plaques formed on any given strain can be isolated an "adapted phage," which multiplies normally on the same strain and forms only rare plaques on the others. Some of these "adapted phages" are host-range mutants but most of them revert to the original nonadapted *Vi* phage II upon a single transfer on strain A. The modification resulting in "adaptation" of the *Vi* phages is thus, in most cases, a phenotypic change induced by the host. Analysis of these cases has shown that *Vi* positive strains which induce such phenotypic changes are lysogenic and differ by the prophage they carry. These prophages appear to be unrelated to *Vi* phage II. In such strains, therefore, the presence of a prophage not only decreases the sensitivity of a given *Vi* positive strain to a *Vi* phage grown on another strain, but it also determines the capacity of this strain to alter in a specific way the properties of those phages which succeeded in multiplying (Anderson and Fraser, 1955).

Numerous examples of such interference of lysogeny with the multiplication of unrelated phages have now been described in many species. This kind

of phenomenon forms to a great extent the basis of the differences between various strains of the same species as classified by phage typing.

### *C. Lysogenic Conversions*

Other alterations in the characters of the host as a result of lysogenization appear to be so entangled with the lysogenic character than they can be ascribed to the very presence of the prophage. The most striking case is the production of toxin by *Corynebacterium diphtheriae*. It was observed by Freeman (1951) that most of the toxinogenic strains are lysogenic and release phage particles which are active on other nontoxinogenic, nonlysogenic strains. The lysogenic clones which can be isolated by infection are all toxinogenic and the toxinogenic character can be passed from strain to strain by lysogenization (Groman, 1953). The toxinogenic character disappears when lysogeny is lost. A complete correlation is observed between lysogeny and toxinogeny (Groman, 1955). However, such a property appears to be restricted to certain strains of temperate phages of *C. diphtheriae* (Barksdale, 1955; Groman and Eaton, 1955). Experiments by Groman and Eaton also suggest that the ability to confer toxinogeny segregates in crosses between related phages.

The exact nature of the relationship between lysogeny and toxinogeny is not yet clear. Phage and toxin are antigenically unrelated. It seems unlikely that toxinogenic bacteria result from the selection by the phage of pre-existing toxigenic mutants (Groman, 1953). Toxin production is not correlated with vegetative multiplication of phage (Barksdale and Pappenheimer, 1954). The favored hypothesis is that the very presence of some types of prophages confers on certain bacteria the ability to produce a specific protein.

In *B. megatherium*, a case has been reported by Ionesco (1953) in which the presence of a given prophage modifies the colonial morphology. The colonies revert to "normal" after the loss of the prophage.

In *Salmonella*, the presence of certain somatic antigens appears to be controlled by the presence of certain prophages, as shown by Iseki and Sakai (1953). Strains of subgroup E<sub>1</sub> possess O antigens III, XV. They are lysogenic and release phage  $\epsilon$ . Strains of subgroup E<sub>2</sub> possess antigens III, X. They are sensitive to phage  $\epsilon$ . Upon infection of bacteria E<sub>2</sub> with phage  $\epsilon$ , the lysogenic derivatives E<sub>2</sub>( $\epsilon$ ) become antigenically III, XV. The presence of prophage  $\epsilon$  appears therefore to be correlated with the formation of the somatic antigen XV. Likewise, in *Salmonella* group B, the presence of prophage "iota" appears to control the formation of somatic antigen I (Iseki and Kashiwagi, 1955).

These various observations indicate clearly that a prophage may eventually interfere with the biochemical activities of the host cell. Such alterations of the host physiology resulting from the presence of a prophage might possibly

be rather frequent. They can be recognized only by comparison of the properties of lysogenic and nonlysogenic derivatives of the same strain. Moreover the presence of a prophage is likely to be overlooked in cases such as the defective lysogenic strains in which a mutation of the prophage prevents the formation of infectious particles (see Section VI, D). The genetic determinant of any physiological character controlled by a defective prophage would undoubtedly be thought to be, not a prophage, but a bacterial gene.

## VI. CHARACTERS OF TEMPERATE PHAGES

### *A. General Properties*

The general properties of temperate phages may be studied, using standard phage techniques, by infection of a suitable sensitive indicator. Although, upon infection, a certain fraction of the infected bacteria becomes lysogenic, this proportion is generally too low to alter significantly the quantitative analysis of the results. When plated on a sensitive indicator, temperate phages produce, in general, turbid plaques, as a result of the growth of lysogenic clones during the formation of the plaque. The degree of turbidity is, to a certain extent, a measure of the lysogenizing power of the phage under study. For any temperate phage, such properties as adsorption, stability, latent period, and burst size, may thus be determined. They are found to be as characteristic of any given phage, and as variable from phage to phage as among virulent phages. In general, however, temperate phages are found to be more difficult to work with than virulent phages like those of the T series: lysis in liquid medium is rarely complete, high titer stocks more difficult to obtain, the latent period is longer.

Temperate phages are frequently excluded by virulent phages. For example, when lysogenic *E. coli* K12( $\lambda$ ) in which the development of the temperate phage  $\lambda$  has been induced by UV light, are infected with T5 particles,  $\lambda$  is excluded by T5. This is true even if infection with T5 occurs only a few minutes before intracellular appearance of  $\lambda$  mature particles (Weigle and Delbrück, 1951).

Like other phages, temperate phages are composed of DNA and protein. As they are of a smaller size than the T-even phages, they contain less DNA (Smith and Siminovitch, in Lwoff, 1953) and are therefore more resistant to radiations. One important property of temperate phages is their inability, when inactivated by UV light, to kill sensitive bacteria on which they adsorb. Their protein coat appears to be devoid of lethal properties, a condition which is indeed necessary for a phage to be temperate: that is, to be able to establish, upon infection, a lysogenic system.



### *B. Action of Temperate Phages on Bacterial Metabolism*

The action of temperate phages, or their mutants, on sensitive bacteria is very different from that of virulent phages such as T2, T4, and T6. This is not surprising, since it is known that the protein coat of virulent phages, such as T2, has by itself a strong inhibitory effect on all synthesis of sensitive bacteria (French and Siminovitch, 1955), whereas temperate phages inactivated with UV light do not even interfere with bacterial growth. Whether phage development results from infection of sensitive bacteria or from induction of lysogenic bacteria, bacterial growth proceeds without cell division during the latent period of temperate phages (see Fig. 3). The increase in turbidity corresponds to actual syntheses of bacterial substance: oxygen uptake as well as RNA synthesis increase in a manner parallel to bacterial turbidity (Siminovitch and Rapkine, 1952). Not only do normal bacterial syntheses proceed, but induced biosynthesis of enzymatic systems may also be initiated at any time during the latent period (Siminovitch and Jacob, 1952).

Although bacterial syntheses still proceed during the multiplication of temperate phages, phage syntheses take place preferentially. This is shown by varying the rate of supply of either the carbon or the nitrogen source during phage development (Jacob, 1952b). A decrease in food supply may suppress bacterial growth without affecting phage production. For low supplies, the available metabolite is used exclusively for phage synthesis. Only when the food supply is high enough, does the surplus of metabolite become available for bacterial synthesis.

### *C. Genetics of Temperate Phages*

Mutations of temperate phages have been observed in all systems which have been carefully investigated, whether it be with the phages of *B. megatherium* (Murphy, 1952; Ionesco, 1956), *Salmonella* (Boyd, 1951; Levine, 1957), *E. coli* (Bertani, 1953a; Weigle, 1953; Jacob and Wollman, 1954; Kaiser, 1955), or of *Pseudomonas* (Jacob, 1954; Dickinson, 1954). Some mutants differ from the wild type in their plaque morphology, plaque size, or host range, as in the case of virulent phages. Other mutants differ in their lysogenizing power. The most easily recognizable are those which are unable, or almost unable, to lysogenize. These mutants form clear plaques instead of the turbid ones formed by the wild type. They were observed even in the early days of lysogeny (den Dooren de Jong, 1931; Burnet and Lush, 1936) and have since been found in all systems that have been examined. They occur with a rather high frequency ( $1$  in  $10^3$  to  $1$  in  $10^4$ ) and different types can be recognized among them. Most of these mutants are unable to multiply on lysogenic bacteria carrying the wild-type prophage. In the rare cases,

however, mutants have been found which are able to multiply as efficiently on lysogenic as on nonlysogenic derivatives of the same strain (Bertani, 1953a; Lederberg and Lederberg, 1953; Jacob and Wollman, 1953). In view of their ability to overcome the immunity conferred on lysogenic bacteria by a homologous prophage, these mutants may be called virulent mutants (Jacob and Wollman, 1953, 1954).

In certain systems, the frequency of phage mutations can be enormously increased by irradiating both phage particles and sensitive bacteria before infection, as shown by Weigle (1953) with *E. coli* K12 and phage  $\lambda$ . If irradiated cells are infected with irradiated phages, a large fraction of the "inactive" particles are reactivated, among which up to 5 or 10 % produce a pure progeny of mutant type. The same increase in the mutation frequency results also from induction of lysogenic *E. coli* K12( $\lambda$ ) with rather high doses of UV light. No effect is observed after irradiation of the phage alone. A certain increase in the frequency of some mutations has been observed after infection of irradiated bacteria with nonirradiated phage (Jacob, 1954; Hershey *et al.*, 1954). When mutants of temperate phages differ by two or more characters, genetic recombination between these characters may be observed either after mixed infection of sensitive cells or after infection of UV-induced lysogenic bacteria carrying a prophage of one type with particles of the other type. The main features are essentially similar to those which have been observed with virulent phages and the theory of Visconti and Delbrück (1953) can be applied to temperate phages. The average number of rounds of mating is rather low, 0.5 or less (Murphy, 1953; Wollman and Jacob, 1954; Kaiser, 1955). When genetic recombination has been carefully analyzed, temperate phage have been found to exhibit a single linkage group, on which all the known mutations can be mapped. Finally, an increase in recombination frequency between any pair of markers has been observed by exposing to low doses of UV light either the phage particles before infection or the phage-bacterium complex after infection (Jacob and Wollman, 1955).

Genetic recombination between temperate phages and their mutants, whose lysogenizing power is either decreased or lost, allows a genetic analysis of the process of lysogenization (see Section VII, B).

#### *D. Prophage Mutations : Defective Lysogenic Bacteria*

Many of the mutations which affect temperate phages have also been found to occur in the prophage state. Other prophage mutations exist which are of special interest because they prevent the formation of phage particles, and may thus be detected and analyzed only in the prophage state. Strains carrying such mutant prophages are called *defective* lysogenic strains.

Defective lysogenic strains of various species have been isolated, either

after infection of sensitive cells with temperate phages, or among the survivors of normal lysogenic bacteria which have been exposed to heavy doses of UV light (Jacob, 1950; Lwoff and Siminovitch, 1951; Appleyard, 1954b; Jacob and Wollman, 1956). In the cultures of such defective lysogenic bacteria, no infectious particles (or very few) are found. After exposure to UV light, lysis of the culture may or may not be observed. When lysis occurs, its characteristics are the same as with normal lysogenic cells, but phage particles are released by a very small fraction of the population, as low as  $10^{-5}$  or less. The lysogenic character of the defective bacteria is nevertheless clearly demonstrated by the fact that they exhibit the same immunity pattern as the homologous normal lysogenic bacteria. In such a system, the expression of lysogeny remains lethal for the host but is not accompanied by the release of infectious phage particles. If a defective lysogenic strain were isolated from nature, its lysogenic character could well remain unnoticed.

In a defective strain, some lesion of the lysogenic system prevents the formation of mature phage particles. This lesion is generally located on the prophage and not on the bacterial component of the system, since defective bacteria induced and then infected with a homologous phage support the growth of this phage and release mature particles (Appleyard, 1954b; Jacob and Wollman, 1956). When the phage used for superinfection is marked by a variety of genetic characters not only may it be recognized that the defective character is a genetic property of the prophage but this particular mutation may actually be mapped on the prophage linkage group. Genetic analysis of various defective strains indicates that a variety of prophage mutations may be responsible for the defective character. Mutations at different loci appear to interfere with different steps of phage development. After UV induction, prophage development may be initiated, but the lesion prevents one of the steps involved in the completion of mature particles. The rare infectious particles released result from mutations of the defective allele to a normal one (Jacob and Wollman, 1956). Among the few cases which have been analyzed in detail, one implies a block in the morphogenesis of the phage particles. Although all recognizable materials of phage  $\lambda$  appear to be produced after UV induction, one of the reactions leading to their assembling into mature particles is missing. In another case, it is the synthesis of a pooled constituent which appears to be altered, since upon infection of UV-induced defective bacteria with a homologous phage, both normal and "defective" particles (that is, particles able to lysogenize but not to reproduce) are released (Appleyard, 1956). Defective lysogenic bacteria therefore offer the remarkable situation of a phage genetic material which can be indefinitely perpetuated only in the prophage state, since lethal alleles prevent its development into infectious particles.

## VII. LYSOGENIZATION

By definition, temperate phages are able to lysogenize. Sensitive bacteria can respond to infection with temperate phages by different series of events. In a certain fraction of the population, the infecting particles enter the vegetative state. They multiply and the bacteria lyse, releasing a burst of phages (*productive* response). In another fraction, the infecting particles turn to the prophage state. These bacteria survive and produce clones containing lysogenic cells (*lysogenic* response). Exceptionally, in some rare cells the infecting particle disappears. Such cells may either survive without becoming lysogenic (*refractory* response), or die without releasing phage (*lethal* response) (Lieb, 1953).

Under standard conditions, any given temperate phage gives a constant fraction of lysogenic responses upon infection of a given strain of sensitive bacteria. This constant rate of lysogenization could be due to an intrinsic heterogeneity in the phage population, of which only a fraction, generally small, would be able to lysogenize at all (Boyd, 1952). Under another hypothesis, the population of infecting phages would be essentially homogeneous with respect to their lysogenizing power, but each particle would have a small but constant probability, under a given set of conditions, of establishing itself as a prophage instead of entering the vegetative state (Lieb, 1953). As, in a given population of sensitive bacteria, any clone appears identical as far as its behavior toward infection with a temperate phage is concerned, the variability in the bacterial response would be of a *phenotypic* and not of a *genotypic* nature. This is indeed what has been found in all systems which have been analyzed, since the ratio "productive response : lysogenic response" may be modified by changes in the conditions of infection.

Nevertheless, the capacity of a phage to lysogenize is genetically controlled, since mutants can be isolated whose lysogenizing ability is altered or even suppressed (see Section VI, C).

*A. Phenotypic Aspects of Lysogenization*

The frequency of lysogenization can be modified by varying the conditions of infection. The factors which bring about such modifications differ widely, according to the system under study.

After infection of *Shigella dysenteriae* with phage P1, the frequency of lysogenization is strongly affected by temperature (Bertani and Nice, 1954). At 37°C. most of the infected cells give a productive response, and only a few become lysogenic. At 20°C. most of the cells give a lysogenic response. When bacteria are infected and incubated at 37°C., the shift toward lysogenization can be observed even when the cells are brought to 20°C. as late as 20 or 30 minutes after infection. This result suggests that, after infection, the first steps leading to both lysogenization and phage production remain identical for at least 20 minutes.



Another effect on lysogenization has been observed by Boyd (1952) in *Salmonella typhimurium* infected with phage A. When the multiplicity of infection is small (one or less), most of the infected bacteria lyse and produce phage. When the multiplicity of infection is gradually increased, the proportion of infected cells that become lysogenic increases too. When the cells are infected with an average of 10 particles per bacterium, the fraction of lysogenics reaches almost 100 %. Thus, the infecting particles can cooperate in establishing lysogeny. In this system the response to infection is also dependent on cultural conditions (Lwoff *et al.*, 1954). The lysogenization frequency for a given multiplicity of infection can be influenced by such treatments as starvation of the cells or addition of various metabolites and antimetabolites to the cultures. The factors which may shift the responses toward lysogenization or phage production are efficient only during the first 6 or 8 minutes following infection. Only at the end of this period does an irreversible reaction, leading to one or another type of response, take place. Cooperation between infecting particles is also effective during the first minutes following infection (Lieb, 1957). When bacteria are first infected with small multiplicities of phage A, superinfection with high multiplicities of the same phage or its mutants will increase the lysogenic responses only during the first few minutes, but not later.

In the case of *E. coli* K12 and phage  $\lambda$ , analyzed by Lieb (1953), the factors influencing the decision between lysis or no lysis appear to be still more complex. A rapid and apparently irreversible modification seems to be undergone by the infecting particle, leading towards either the vegetative or the prophage state. Among the cells that will not lyse, some give rise to clones which consist exclusively of lysogenic cells, others to clones of sensitive cells; the remaining clones are mixtures of sensitive and lysogenic cells. The proportion of clones that contain only lysogenic cells and also the proportion of lysogenics in the mixed clones can be increased by increasing the multiplicity of infection or by decreasing the temperature of incubation for one hour after infection. This suggests that those particles which have not undergone the transition to the vegetative state do not divide before becoming a prophage and at each bacterial division are randomly distributed among the daughter cells. During this period, the phage material is very sensitive to temperature: it can be inactivated by incubation at 44°C. Only after the end of the first hour does the phage material behave as a prophage: it divides in harmony with the cell and exhibits the same temperature sensitivity as the host.

### *B. Genetics of Lysogenization*

In temperate phages, various mutations occur which alter or even suppress the capacity for lysogenization exhibited by the wild type. Some of these

mutations seem to affect the stability of the prophage in the course of bacterial division. Others affect the very process by which the phage is converted into prophage. The latter mutants, when plated on sensitive bacteria, form clear plaques, in contrast with the turbid centered plaques formed by the wild type. Genetic analysis of these so-called *clear* (*C*) mutants in both phage A of *S. typhimurium* (Levine, 1957) and phage  $\lambda$  of *E. coli* K12 (Kaiser, 1957) has brought valuable information about the lysogenization process. The *C* mutants can be subdivided into three classes ( $C_1$ ,  $C_2$ , and  $C_3$ ) according to their phenotype, that is, the aspect of the plaques they form. All of the *C* mutations appear to be located on a short region of the phage linkage group. Moreover, the  $C_1$  mutants are all located in a cluster on a segment of the *C* region ( $C_1$  segment). Likewise, mutants of the  $C_2$  and  $C_3$  phenotypes are located, also in clusters, on a  $C_2$  and a  $C_3$  segment, respectively, of the *C* region.

Whereas infection of sensitive bacteria with a given *C* mutant leads to no, or very little, lysogenization, mixed infection with pairs of mutants belonging to different types ( $C_1 + C_2$ ,  $C_1 + C_3$ ,  $C_2 + C_3$ ) results in a rate of lysogenization analogous to that found with the wild type. Lysogenic bacteria isolated after such mixed infections carry either one type of prophage, in the case of *Salmonella*, or both types, in the case of *E. coli* K12. On the contrary, mixed infection with mutants of the same group does not increase the lysogenization frequency. This situation, which is similar to that analyzed by Benzer (1955) in the case of  $r_{II}$  mutants of T2 is equivalent to a case of "pseudo allelism." Mixed infection with two mutant types simulates a heterozygous diploid in the *trans* configuration. The application of the phenotype test to pairs of *C* mutants leads to the division of the region into three functionally separable segment each segment probably controlling a different reaction leading to the prophage state. The nature of these reactions, as well as their sequence, is not yet known.

### VIII. GENETIC DETERMINISM OF LYSOGENY

A lysogenic bacterium possesses and transmits to its progeny the information necessary for synthesizing a definite type of phage particles. When the lysogenic system has been established, as a consequence of infection of a sensitive cell by a given type of temperate phage, the phage liberated is identical to the original type. The stability of the lysogenic character, once established, implies an efficient mechanism for the inheritance of this character. Schematically, such a mechanism can be ensured, either by a specific process of replication and segregation of the prophage itself as a nuclear structure at each cell division, or by a random process, provided the number of prophages is high enough so that the probability for any daughter cell not to receive at least one copy of it at each division is negligible.

### *A. Limited Number of Prophages per Cell*

It is possible by indirect means to estimate the number of prophages present in a lysogenic cell. On the one hand, it is known that when sensitive bacteria are mixedly infected with different ratios of two mutant phages, the same ratios are found in the progeny. On the other hand, it is known that when UV-induced lysogenic bacteria are infected with a mutant phage, the proportion in which the infecting type is found in the progeny increases as a function of the multiplicity of infection. Assuming that after UV induction a prophage plays the same role in phage multiplication as an infecting particle, the number of prophages must be equal to the average multiplicity of infection for which a ratio of 1 : 1 is to be found in the progeny. The number of prophages per bacterium may thus be evaluated as averaging three, which is in good agreement with the average number of nuclei per bacterium in a growing population (Jacob and Wollman, 1953). Similar conclusions have also already been reached by Bertani (1953b) from experiments in which non-inducible lysogenic bacteria were infected with a mutant phage and the phage production analyzed as a function of time.

Lysogenic bacteria, therefore, appear to contain one prophage per nucleus. Another line of evidence comes from the incompatibility observed between related prophages (Bertani, 1953a). The fact that, in most cases, lysogenic bacteria are able to carry only one prophage of the same type, whereas they may easily carry various unrelated prophages, suggests that, for a given type of prophage, there must exist in a bacterium only a very small number of specific sites saturated by the prophages.

### *B. Chromosomal Location of the Prophage*

The decision as to whether the prophage is a cytoplasmic or a chromosomal structure can only be obtained through experiments of genetic recombination. Such experiments are made possible by the existence, in strain K12 of *E. coli*, of sexual processes discovered by Tatum and Lederberg (1947). Wild-type strains of *E. coli* K12 are lysogenic and carry a prophage called  $\lambda$ . Nonlysogenic clones can be isolated by treatment such as exposure to heavy doses of UV light (Lederberg, 1951). The genetic behaviour of the lysogenic character may therefore be analyzed in crosses between lysogenic ( $ly^+$ ) and nonlysogenic ( $ly^-$ ) bacteria.

The first experiments designed to analyze the behaviour of the  $ly$  character were carried out by Lederberg and Lederberg (1953) and by Wollman (1953), using a system in which only one recombinant is formed per million of each parental type. Although such a low frequency of recombination makes difficult any quantitative work, valuable information was obtained. In certain crosses, the  $\lambda$  lysogenic character segregates among recombinants and

appears to be linked with another genetic character which controls galactose fermentation (*Gal*). Such results support the hypothesis that the lysogenic character is under the control of a nuclear determinant. That this determinant is the prophage itself appears from experiments involving two lysogenic strains, each one carrying a different  $\lambda$  mutant. As shown by Appleyard (1954a), in such a cross the genetic markers of the prophage itself appear to be linked to the *Gal* character. However, in certain crosses, anomalies were observed in the inheritance of the *ly* and *Gal* characters (Wollman, 1953; Appleyard, 1954a; Frédéricq, 1954).

Recent advances in our knowledge concerning bacterial recombination (Wollman *et al.*, 1956) have allowed a more accurate investigation of the genetic determinism of lysogeny (Jacob and Wollman, 1957). In *E. coli* K12, bacterial conjugation may be visualized as the oriented injection of a linear segment carrying linked genes from donor (or Hfr) into recipient (or F<sup>-</sup>) bacteria. In crosses between nonlysogenic donor Hfr *ly*<sup>-</sup> and lysogenic recipient F<sup>-</sup>*ly*<sup>+</sup> the nonlysogenic character is transferred and segregates among recombinants as any other genetic character. It can be located at about 15 recombination units from *Gal*.

In crosses where both donor and recipient parents are lysogenic, each one carrying a different  $\lambda$  mutant, the Hfr prophage segregates among recombinants exactly as the nonlysogenic character in the previous cross. The conclusions can therefore be drawn that  $\lambda$  prophage occupies a definite position on the bacterial chromosome. In the two crosses previously mentioned, it behaves exactly as any bacterial genetic character.

However, the situation is quite different in crosses between Hfr *ly*<sup>+</sup> and F<sup>-</sup>*ly*<sup>-</sup>. The *ly*<sup>+</sup> character of the donor is not transmitted to recombinants. This is due to the fact that whenever a donor injects into a nonlysogenic recipient a segment of chromosome carrying the  $\lambda$  prophage, this prophage develops in the recipient cell. The recipient cell lyses and releases one hundred phage particles. This phenomenon is called *zygotic induction*. Whenever  $\lambda$  prophage is transferred to a nonlysogenic recipient, it behaves as a lethal character immediately expressed. As a consequence, those zygotes which have received a piece of the Hfr genetic material carrying  $\lambda$  are destroyed. Nothing similar can be demonstrated in crosses involving a lysogenic F<sup>-</sup> parent, whether or not the Hfr parent is lysogenic. The presence of a prophage confers to the F<sup>-</sup> cell an immunity against zygotic induction.

Thus, experiments of bacterial recombination demonstrate that lysogeny is under the control of a nuclear determinant, the prophage itself, which is located at a specific site of the bacterial chromosome. However, the phenomenon of zygotic induction indicates that the prophage does not behave exactly as a normal genetic component of the host.



### *C. Specific Locations of Different Prophages*

The fact that, whereas a lysogenic bacterium most frequently carries only one prophage of a given type, it may carry several unrelated prophages, suggests that there exists a specific bacterial site for each type of prophage. The location of fourteen different prophages has been investigated by means of *E. coli* K12 crosses (Jacob and Wollman, 1957).

Half of these prophages are UV-inducible. Although each of them exhibits a different immunity pattern, the homologous phages appear to be serologically related and able to undergo genetic recombination on mixed infection of sensitive bacteria. All these UV-inducible prophages have been found to be linearly arranged on a segment of the bacterial chromosome close to the *Gal* character. On this segment, each of the prophages occupies a definite location. Moreover, in crosses  $Hfr\ ly^+ \times F^- ly^-$ , zygotic induction is observed with every prophage, although at different degrees depending on their specific location.

On the contrary, UV-noninducible prophages appear to be located on other segments of the bacterial chromosome. No zygotic induction occurs with any of them.

It can therefore be concluded that each of these fourteen prophages occupies a specific location on the chromosome of *E. coli* K12. Ability to lysogenize thus appears to involve a genetic property of the phage, as well as the existence on the chromosome of the host of a specific site for the homologous prophage. Mutations of the host could thus be imagined which would involve the loss of its ability to be lysogenized. Such mutations have not as yet been observed.

Work on doubly or triply lysogenic strains for phage P2 has led Bertani (1956) to conclude that noninducible prophage P2 could occupy, with equal probabilities, one of three different locations.

From the locations found for inducible prophages on the one hand, for noninducible ones on the other hand, UV inducibility could be a property, not of a given phage as such, but perhaps of the chromosomal segment on which certain types of prophages happen to have their location.

### *D. Nature of the Prophage*

Our knowledge about the chemical nature of the prophage or its physical relationship to the bacterial genetic apparatus is still a matter of speculation.

Since lysogenic bacteria obtained after infection with a temperate phage produce phage particles identical with the original type, the prophage must have the same genetic potentialities as the original phage. From experiments by Hershey and Chase (1952), it is apparent that, in phage reproduction, these potentialities are borne by the DNA of the infecting particle. Several

lines of evidence suggest that the prophage is indeed a DNA structure. First of all, the abilities of temperate phages either to multiply or to lysogenize are equally sensitive to UV inactivation or  $P^{32}$  disintegration both before and after infection of the sensitive host (Garen and Zinder, 1955; Stent and Fuerst, 1956). Like vegetative phage, the prophage appears to evolve, upon lysogenization, from the DNA of the infective particle. Moreover, lysogenic bacteria do not contain any recognizable antigen of the homologous phage (Miller and Goebel, 1954). Finally, the prophage has recently been shown to exhibit the same sensitivity to  $P^{32}$  decay as the homologous infectious particle (Stent *et al.*, 1957). This result would indicate that the prophage contains the same amount of phosphorus atoms and, probably, of DNA as the genetic material of the free phage.

Another important point which still remains unclear is what makes the basic difference between the prophage, which replicates together with the nuclear structures of the bacterium, and the vegetative phage, whose multiplication brings about the destruction of the host. Such a difference in the behavior of the two types of structures can hardly be ascribed to a difference in their *nature* but rather in their *activity*. In a lysogenic bacterium, the prophage (probably DNA) replicates, but apparently does not synthesize any phage protein. During the vegetative phase, not only does the genetic material replicate, but also phage proteins are produced. This is the only qualitative difference so far known between the lysogenic and the vegetative phase of the life cycle of a phage.

Obviously, the chromosomal location of the prophage explains most of the known facts relevant to lysogeny. A lysogenic bacterium may be visualized as possessing the genetic material of a phage located at a specific site of the bacterial chromosome. The two integrated structures replicate as a whole. Different lines of evidence suggest that the prophage is added to, and not substituted for, a homologous segment of the host chromosome. It does not seem to be inserted in the axis of the bacterial chromosome, but bound to it in some yet unknown way (Jacob and Wollman, 1957).

The presence of this added genetic material provides the host cell with some specific properties, such as ability to make phage and immunity against homologous particles. When the prophage is inducible, it also determines a high sensitivity to radiations and inducing compounds. In some cases, this new structure may also modify some functional property of the host, such as toxin or antigen syntheses, which at first sight appear to be unrelated to phages.

## IX. TEMPERATE PHAGES AS GENETIC VECTORS

The genetic analysis of lysogeny has unraveled the existence, in lysogenic bacteria, of an intimate connection between the genetic materials of a

temperate phage and of the host cell. In addition, temperate phages may be involved in the mechanisms of genetic exchange in bacteria.

In 1952, it was reported by Zinder and Lederberg that, in *Salmonella typhimurium*, genetic characters could be transferred from bacteria of one strain acting as a donor to bacteria of another strain acting as a recipient by exposing the recipient to culture filtrates of the donor. The agent present in the preparations and responsible for the transfer of genetic characters was later identified by Zinder (1953) as a temperate phage released by the donor and active on the recipient.

Transduction, therefore, is the mechanism by which a temperate phage transports a fragment of genetic material from a donor bacterium, on which it has multiplied, to a recipient bacterium, which it infects. Among the recipient cells which survive infection, rare individuals acquire new genetic properties. In most cases, transduction is not specific in the sense that any character of the donor can be transmitted by a small fraction of the phage particles ( $10^{-5}$  or  $10^{-6}$ ). It is independent of lysogenization by the transducing particles, since lysogenic recipients can be transduced by clear mutants unable to lysogenize (Zinder, 1953). It seems likely that, in the course of phage maturation, in addition to the phage genetic material, small segments of the host chromosome can be incorporated at random into the protein coat of maturing phage particles.

Since its original discovery in *Salmonella typhimurium*, transduction has now been described in several bacterial strains of *Salmonella* (Zinder, 1955), *E. coli*, and *Shigella* (Lennox, 1955). Transducing activity, however, is restricted to a limited number of temperate phages active on a given strain.

When characters are closely linked, they may be transduced simultaneously (Stocker *et al.*, 1953; Lennox, 1955). This method allows an accurate analysis of the fine structure of small segments of the bacterial chromosome (Demerec *et al.*, 1955). In *E. coli* K12( $\lambda$ ), the lysogenic character itself may also be transduced, together with the closely linked character *Gal* by means of unrelated transducing phages (Jacob, 1955). This not only confirms the chromosomal location of the prophage, but also shows that a phage particle may contain the entire genome of another unrelated phage.

A completely different type of phage-mediated transfer of genetic characters has been recently described by Morse and associates (1956). In *E. coli* K12( $\lambda$ ), prophage  $\lambda$  is located near characters controlling galactose fermentation (*Gal*). After induction by UV light of *Gal*<sup>+</sup> lysogenic K12( $\lambda$ ), a small proportion ( $10^{-6}$  or  $10^{-7}$ ) of the phages released may transmit the *Gal*<sup>+</sup> character upon lysogenization of a *Gal*<sup>-</sup> nonlysogenic K12. Lysogenic *Gal*<sup>+</sup> clones thus obtained exhibit a remarkable property: they segregate *Gal*<sup>+</sup> as well as *Gal*<sup>-</sup> progeny; they therefore possess both *Gal* alleles and for this reason have been called *heterogenotes*. When heterogenotes

are induced, at least 50 % of the released phages are able to transduce the *Gal*<sup>+</sup> character. Quantitative measurements lead to the conclusion that, upon induction, both the genetic material of the phage and the *Gal* character multiply as a single unit (Weigle, 1957).

Contrary to transduction in *Salmonella*, for instance, transduction of the *Gal* character by phage  $\lambda$  is very specific: the only character hitherto known to be transduced by phage  $\lambda$  is precisely that character to which  $\lambda$  prophage is linked. This type of transduction clearly illustrates how intimate is the association of a prophage with the genetic material of the host, since it may even persist during the vegetative multiplication. It is an intermediary situation between the other cases of transduction where the role of the phage appears to be only passive, and those situations of lysogenic conversions, such as toxin production, where the genetic determinant of the character appears to be the prophage itself.

#### X. LYSOGENY AND LATENCY OF VIRUSES

Lysogeny corresponds to a remarkable situation whereby the genetic material of a virus is incorporated to that of the host and behaves as a cellular constituent. Either spontaneously or as a result of the action of mutagenic or carcinogenic agents, this cellular constituent will eventually develop into a pathogenic form, whose multiplication brings about the destruction of the host cell and the liberation of a crop of infectious particles. Infection of sensitive cells may in turn give rise to new lysogenic systems. The cycle of phage in such lysogenic systems with its three stages (infectious, vegetative, and prophage) is schematically represented in Fig. 6.

The case of lysogenic bacteria has often been brought close to that of latent viral infections in plants or animals (Lwoff, 1953; Jacob, 1954). Numerous cases of such latent infections are now known and have been recently reviewed (Shope, 1950; Koprowski, 1952; Smith, 1952; Schlesinger, 1953). They correspond to a wide variety of situations. In some cases, latency occurs after a known infection with a given virus. Sometimes, this infection is not followed by pathological symptoms, but the persistence of the virus is evidenced either by the possibility of recovering active virus from the infected organism, by the presence of specific antibodies, or by interference with a subsequent infection with a related virus. Sometimes, infection may be followed by such lesions as the production of a tumor, but no, or very little, infectious virus can be recovered from the infected organism. In other cases, whether after infection or even in the absence of any known infection, pathological symptoms do not appear unless the organism is submitted to environmental changes, either physiological or brought about by the action of external physical or chemical agents. It thus appears that what is generally



included under the name of latent viral infection may correspond to a variety of types of relationships between host and virus, the analysis of which has been made extremely difficult in most cases by the complex nature of the host system and by the limitation of the procedures of titration of the virus.

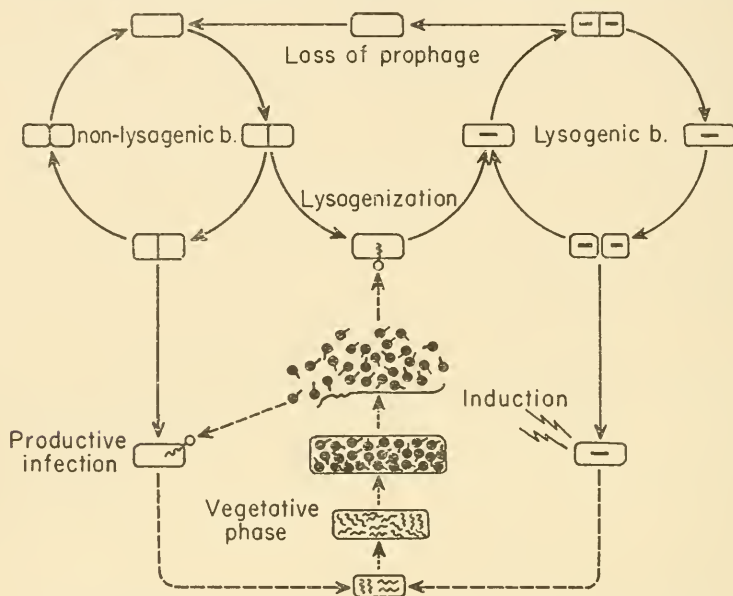


FIG. 6. Diagrammatic representation of lysogeny. (After Lwoff, 1953.)

In view of the fact, however, that phage research itself has been so fruitful a model for the study of animal and plant viruses, it is tempting to think that situations which have been analyzed in the field of bacteriophage will find their counterpart in other fields of virology. More especially, since we know that the prophage may in certain instances modify in a specific way some function of the host, it might be helpful to bring together lysogeny and those cases of tumors where viruses appear to be the responsible agents. It is likely that although many of the cases of latency will be finally ascribed to complex interactions between viruses and the different cellular systems present in metazoa, some of them will be shown to represent cases comparable to lysogeny, that is, cases whereby the genetic material of the virus behaves as an integrated structure of the infected cell. Under this definition a *provirus*, like a prophage, is a noninfectious form of a virus which exists and is perpetuated in stable intracellular association with the structures of the host cell. Evidence for the existence of a provirus stage therefore will not be obtainable until the relationship between viruses and their hosts at the cellular level is better understood.

There is one instance, however, in which the relationship that exists between a virus and a higher organism parallels to an amazing extent the situation which has been established in the case of lysogenic bacteria. This is the classic case of CO<sub>2</sub> sensitivity in *Drosophila*, which has been extensively studied by L'Héritier, and his group (L'Héritier, 1954). The factor responsible for CO<sub>2</sub> sensitivity has all the properties of a virus, and can be transmitted by injection from CO<sub>2</sub>-sensitive flies to normal individuals. Inheritance of CO<sub>2</sub> sensitivity in *Drosophila* is one of the classic examples of maternal inheritance, since stable CO<sub>2</sub> sensitivity is only transmitted in crosses in which the females bear the character. In such "stabilized" individuals, the virus appears to be integrated in a noninfectious form and is present in every single egg laid by the female. Although stabilized lines are very reminiscent of lysogenic bacteria in many of their properties, the inheritance of the character appears to be better explained by a cytoplasmic rather than by a nuclear localization of the provirus.

Despite this difference, the factor responsible for CO<sub>2</sub> sensitivity in *Drosophila* appears to have all the properties of a provirus. It therefore seems probable that other known or yet unknown cases of latency of viruses of animals or plants will also prove to be a reflection of the existence of a provirus state. The recent developments made in the methods of growth of animal viruses in pure cell cultures (Dulbecco and Vogt, 1953; Puck *et al.*, 1956) show that, for this class of viruses at least, this possibility is not remote.

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# Chapter X

## Radiobiology of Bacteriophage

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### I. INTRODUCTION

#### A. Introductory Remarks

We shall consider experiments and hypotheses based on irradiation of

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phage with three types of agents—(1) ultraviolet light; (2) decay of incorporated radiophosphorus (“suicide”); and (3) ionizing radiations (usually X-rays). At times it may appear that the reviewer has forgotten that the primary aim in employing radiation in the study of phage is to elucidate the *normal* state of affairs. However, almost all experiments involving the irradiation of phage have raised far more questions than they have answered. This has resulted in the situation that there now exists, a “radiobiology of bacteriophage,” a collection of observations and hypotheses arising *from* irradiation experiments, leading no one knows where, but selfishly demanding an explanation. This review will confine itself primarily to those issues which fit this definition of “phage radiobiology.”<sup>1</sup> In so doing, it will not only avoid treading upon the areas covered by other chapters in this volume, but will permit the detailed treatment of the subject demanded by its inherent complexity.

Each of the radio-agents inactivates phage in the sense that a fraction of the particles in a population exposed to the agent is rendered incapable of multiplying. It is likely that each of these agents exerts its lethal effect in large part by damaging the deoxyribose nucleic acid (DNA) of the phage particle. This conclusion is more fully justified in another chapter (Chapter V, Volume I); here we simply indicate why one might expect this to be so.

Most of the ultraviolet light experiments to be discussed were performed with a low-pressure mercury vapor lamp. Such equipment emits wavelengths which are efficiently absorbed by DNA and to which protein is relatively transparent.

Justification for the tentative conclusion that X-rays exert part of their lethal effect on DNA comes simply from the observation that DNA comprises about one-half of the phage particle, coupled with the well-known lack of specificity of X-ray effects on biological molecules. A consideration of phage structure in more detail makes it seem likely that a *major* part of the lethal effects of X-rays is due to damage to DNA. Work of Herriott (1951) and of Hershey and Chase (1952) leads to the picture that the protein (which comprises the other half of the mass of the phage particle) serves as an “overcoat” for the DNA. Its primary function seems to be to encase the DNA and to facilitate its penetration into the bacterial cell. The first function, while perhaps vital, might be supposed to be easily fulfilled; the second function seems to be dependent on only a small part of the protein coat comprising the “tail” of the phage particle.

It would be surprising indeed if the transmutation of phosphorus to sulfur within the phage DNA with its attendant release of energy did not exert a lethal effect on the DNA. Indeed, we shall see later that most, if not all, of the lethal effect of the decay of incorporated  $P^{32}$  is due to damage in DNA.

<sup>1</sup> The facts and problems of phage radiobiology as they appeared in 1951 have been well stated by Luria (1955).

*B. Target Theory*

Modifications of one basic experiment serve as the basis for most investigations in the effects of irradiation on phage. This basic experiment is to measure the survival of some property of the phage as a function of increasing dose of radiation. The simplest such dose-response is well illustrated by the loss of the ability of phage to make a complete cycle of infection, terminated by the release of more infective phage. For both X-rays and suicide (and sometimes for ultraviolet light) such responses are "one-hit." That is, infectivity decreases exponentially as a function of dose, and this exponential response holds over the entire dose range. Equation (1) characterizes such a response.

$$S/S_o = e^{-k_1 D} \quad (1)$$

where  $S_o$  is the original number of infective particles,  $S$  is the number remaining after some dose of radiation  $D$ , and  $k_1$  is a constant which is characteristic of the phage strain and the conditions under which it is irradiated. In many cases the dose is applied at a constant rate so that we can write

$$S/S_o = e^{-k_2 t} \quad (2)$$

where  $t$  is a unit of time, and  $k_2$  is a constant which depends on  $k_1$  and on the rate of application of the dose. It is common practice to plot a "survival curve" as  $\log_{10}(S/S_o)$  versus  $t$  (Fig. 1). The resulting plot is a straight line, the slope of which is a measure of the "sensitivity" of the phage. When the survival of infectivity has reached 0.37,  $k_2 t$  equals 1, and we say that on the average there has been one "hit" per particle. On occasion we shall use the survival equation in the form

$$S/S_o = e^{-r} \quad (3)$$

where  $r$  is the number of hits per particle which inactivate the property of infectivity.

In so defining a "hit" we are adopting a form of "target theory" as a framework for our subsequent thinking. In this model we imagine that darts are flung at random at a number of targets of equal size. The darts correspond to quanta of radiation, the targets to phage particles. A dart will "stick" to a target which it strikes with a probability  $\alpha$ ; i.e., a quantum of radiation impinging upon a phage particle will be effective with a probability  $\alpha$  called the "quantum efficiency." This regime leads to a Poisson distribution of darts stuck into targets (or hits upon phage particles). For any average number of hits  $r$  there will be a fraction of particles  $e^{-r}$  which are not hit at all, a fraction  $re^{-r}$  which are hit once, a fraction  $r^2 e^{-r}/2$  which are hit twice, and, in general, a fraction

$$F = r^x e^{-r} / x! \quad (4)$$

which have been hit  $x$  times.



Chapter V, Volume I, should be referred to for a somewhat fuller discussion of target theory and of the factors which modify the constant  $k_1$ .

These brief remarks on the three radio-agents and the theoretical framework within which they will be considered must suffice as introduction to this review. From this point on, our purpose will be best served by a consideration of one of these agents (ultraviolet light) with emphasis of its effects on one or the other of a pair of closely related phages (T4 or T2).

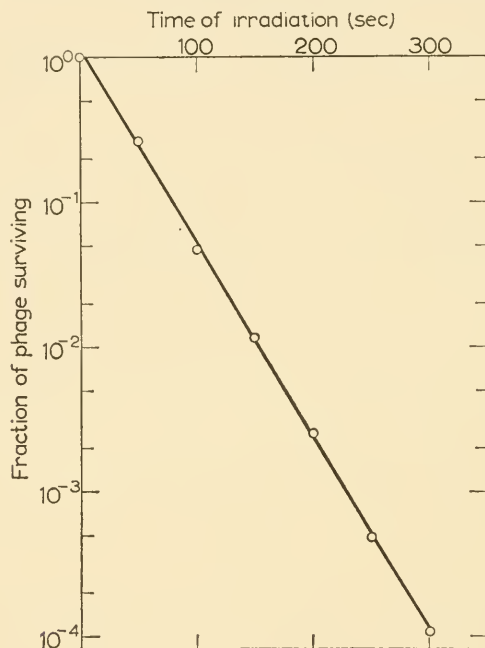


FIG. 1. A "one-hit" survival curve. The logarithm of the fraction of phage retaining the ability to form plaques as a function of the dose of X-rays is linear. The data are for the survival of T6 exposed to soft X-rays in concentrated broth.

With this system the basic problems of radiobiology of phage have been raised; within this system some of these problems show promise of being answered. By means of this system the reader will become acquainted with a way of thought which at present appears the most promising for analyzing the effects of radiation on bacterial viruses.<sup>1</sup> Following this baptism, we shall look at the effects of other agents, and the responses of other phages. The

<sup>1</sup> Perhaps one could equally well introduce the problems of phage radiobiology by detailing our knowledge of the effects of ultraviolet light on the phage  $\lambda$  (see especially Jacob and Wollman, 1955 and Kellenberger and Weigle, 1958). The present choice was to some degree governed by the author's greater familiarity with the T-phages.

framework of notions generated by our consideration of ultraviolet light acting on T4 or T2 will serve as a background against which to view the other agents and other phages.

## II. ULTRAVIOLET IRRADIATION OF T-EVEN BACTERIOPHAGE

### *A. Irradiation of T4*

#### *1. Inactivation of Infectivity*

It is usual to define a surviving particle as one which can produce a visible plaque under the standard conditions for phage assay (see Adams, 1950). The advantage of this definition is the convenience of the plaque-assay method. (A potential disadvantage may be that in extreme cases a heterogeneity in size of plaques among the survivors may lead to some "plaques" being overlooked.)

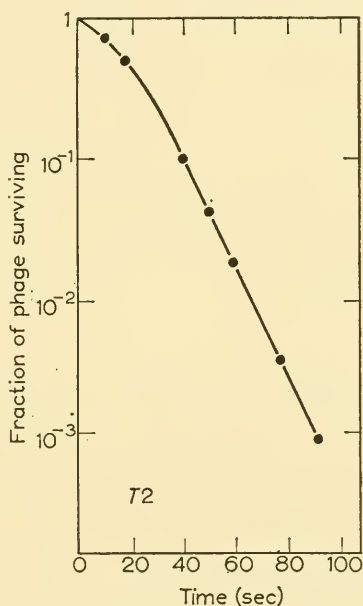


FIG. 2. An ultraviolet light survival curve for T2. A plot of the log fraction of phage surviving versus time of irradiation usually shows a slight initial downward concavity for T2 and T4. This figure is reproduced from Benzer *et al.* (1950) by permission of the California Institute of Technology.

In practice, a survival curve of T4 may fail to be strictly exponential. The curve may show a slight shoulder, i.e., an initial downward concavity (Fig. 2). The extent of the shoulder has been found to depend on the wavelength of ultraviolet light employed (Tessman, 1954). No convincing explanation for the shoulder has been offered; Doermann and Chase (personal

communication), however, consistently get one-hit survival curves with T4 which has been extensively purified by differential centrifugation. This suggests that ultraviolet light may have a reactivating effect on a fraction of the particles in the stock which have been rendered inactive by some "impurity" in the preparation. If such is the case, it must be assumed that the impurity is associated with the DNA of the phage, since phage irradiated after injection of the DNA into the host cell may also show a shoulder (see, for instance, Lennox *et al.*, 1954). Whatever its origin, the shoulder is sufficiently small that for most purposes it can be neglected.

## 2. Multiplicity Reactivation

*a. Definition and the Luria Hypothesis.* In practice, an additional anomaly in the survival curve of T4 is encountered at high doses; the plaque-forming ability seems to become relatively resistant to ultraviolet light. Luria (1947) showed that this is the result of the infection of the same bacterial cells on the assay plate by two inactive particles. The inactive particles are able to cooperate to produce active phage, and hence a plaque. Luria (1947) suggested the following interpretation of this cooperation between inactive particles (multiplicity reactivation). Each phage particle, he supposed, is composed of a number,  $n$ , of equally sensitive units. Each unit is different from the others, and a bacterium will produce phage only if it is infected by particles which among themselves possess a complete set of undamaged units. For cells infected with exactly  $k$  phages, the probability of yielding phage is given by the expression

$$[1 - (1 - e^{-r/n})^k]^n. \quad (5)$$

We shall see that this expression is not a sufficient description of the requirements for multiplicity reactivation in T4. However, the notion of units within a phage each of which is ultraviolet-sensitive marks the beginning of an important phase in radiobiology of phage. This is the notion, explicit in the Luria hypothesis, that the survival curve for single phage particles might meaningfully be written as

$$S/S_o = e^{-(f_1r + f_2r + \dots + f_nr)} \quad (6)$$

where the  $f$ 's are the fractions of the total phage-lethal hits which inactivate target 1, target 2, . . . target  $n$ . The multiplicity reactivation experiments tried to count the number  $n$  of targets. Subsequent work has tried and in part succeeded in determining the nature of the various targets. Thus, the experiments and the hypothesis of Luria were the stimulus for a line of investigation which is now beginning to contribute information on the structure and functional properties of phage.

*b. The Test of the Luria Hypothesis.* In practice one cannot generally work with a population of cells all of which have been infected by the same

number of phage particles. Equation (5) can be modified (Luria and Dulbecco, 1949) to describe theoretically the probability of phage production by the multiply infected members of a bacterial population upon which phage have been randomly (Poisson) adsorbed.<sup>1</sup>

$$y = \frac{\sum_{k=2}^{\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n}{1 - (x + 1)e^{-x}} \quad (7)$$

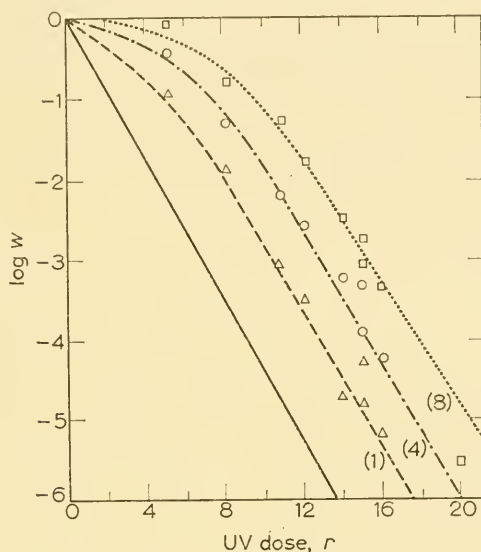


FIG. 3. Multiplicity reactivation of ultraviolet-irradiated Vi-Phage II of *Salmonella typhi*. Values for the survival of multicomplexes ( $w$ ) plotted against the dose in phage-lethal hits ( $r$ ). The solid line gives the survival of the phage and the other curves are those of  $\log y$  as a function of  $r$  for  $n = 4$  and for  $x$  as indicated at the foot of each curve [see Equation (7)]. The figure is from Bernstein (1957).

For a given type of phage, the number of units,  $n$ , is presumed constant, so that  $y$  is dependent on two variables, the average multiplicity of infection,  $x$ , and the average number of hits per phage,  $r$ . At doses so high that essentially no productive cells have more than the minimal number of units required to make phage,  $y$  depends on dose in the same way as does the survival of a single phage particle. That is

$$y_{r \rightarrow \infty} = Be^{-r} \quad (8)$$

<sup>1</sup> The adequacy of the assumption of a Poisson distribution of phages upon cells has been challenged (Cairns and Watson, 1956) on the grounds that one cannot neglect the small number of cells in the culture which are considerably larger than average. Harm (1956), however, found identical results in multiplicity reactivation experiments, using either standard cultures or cultures in which the usual variability in cell size had been greatly reduced by differential filtration.



where  $B$ , the zero-dose extrapolate of the exponential portion of the curve, is a function of  $x$  and  $n$ . Thus, a "critical test" (Dulbecco, 1952a) of the hypothesis consists of the following: (1) the slope of  $\log w$  (the experimentally measured survival of multiply infected cells) versus  $r$  should for large values of  $r$  be constant, independent of  $x$ , and equal to the slope found with single phage particles, and (2) the back extrapolate to zero dose of the linear portion of the plot of  $\log w$  versus  $r$  should for each value of  $x$  give the same estimate of  $n$ .

Multiplicity reactivation in Vi-phage II of *Salmonella typhi* (Bernstein, 1957) is in agreement with the hypothesis (Fig. 3). Significant departures from the hypothesis are found for T4, however.

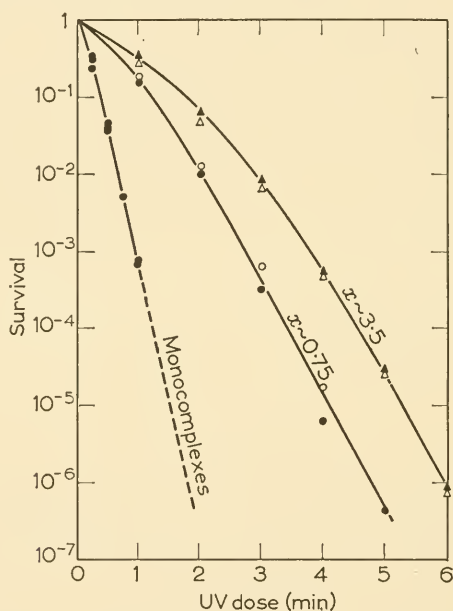


FIG. 4. Multiplicity of ultraviolet-irradiated T4 in *Escherichia coli* B. The data (summarized qualitatively by the smooth curves) are in agreement with the idea that 60 % of the phage target is completely reactivated in multiple infection; the remaining 40 % behaves as three "Luria units." The figure is adapted from Harm (1956).

*c. A Modification of the Luria Hypothesis.* For T4 the plot of  $\log w$  versus  $r$  at high dose has a slope which is significantly less than the slope for the survival of a single particle (Harm, 1956). Harm has pointed out that an explanation of this departure from the Luria hypothesis can be obtained by assuming that the radiosensitive material of T4 is divided into two "parts." One part is reactivated in multiple infection with an efficiency so high that we shall ignore (for the moment) any small influence it might have on the curve; the other part is composed of a small number of units which behave

in a formal sense like Luria units. The total sensitivity of these units ("vulnerable centers," Barricelli, 1956) determines the final slope; their number determines the zero-dose extrapolate.

This theory is obviously similar to the original Luria hypothesis but adds one parameter for the fraction of phage lethal hits which affect vulnerable centers (the formal equivalent of Luria units). Such a two-parameter theory gives a satisfactory fit to the T4 data (Harm, 1956) (see Fig. 4). Within the terms of this notion, the data indicate that 40 % of the total T4 target is composed of three vulnerable centers; the remaining 60 % is reactivated with an efficiency so high as to have no appreciable influence on the curves. [Barricelli (personal communication) has pointed out that the slight decrease in final slope with increasing multiplicity, which, whether significant or not, is a feature of Harm's curves, can be explained on the assumption that the highly reactivable 60 % of the phage is less than perfectly reactivated at high doses.]

The challenge of multiplicity reactivation experiments with T4 becomes that of identifying the three vulnerable centers and the nature of the remaining highly reactivable portion of the radiosensitive target. We proceed with the story having in mind the following conjecture: the three centers represent regions of the phage genetic structure which must perform functions essential for multiplication and recombination of the vegetative phage; the remaining 60 % of the damages occur in regions which need not function until after genetic recombination has occurred. Recombination, in those cells where it can occur, is (almost) always able to reconstitute a genetic structure free of damages. This is essentially the hypothesis put forth by Barricelli (1956).

### 3. Cross Reactivation

*a. Definition.* We define the phenomenon and pose the problems related to it by describing the following (not completely hypothetical) experiment: Genetically marked phages are irradiated to various survival levels. Some phages from each irradiated sample are then tested for their ability to contribute the genetic marker (or markers) to live progeny phages when they are adsorbed to bacteria along with nonirradiated phages of complementary genotype. For a given marker, the "ability to contribute" is characterized by two measurements: (1) the fraction of the irradiated phage which succeeds in transmitting the marker into at least one particle in the progeny; and (2) for those particles which *do* make a contribution, the number of progeny particles which possess the gene derived from the irradiated parent. In such an experiment, the ability to contribute in both senses decreases with increasing dose (Luria, 1952; Doermann *et al.*, 1955).

Within the framework of the hypothesis for multiplicity reactivation stated above, a cross-reactivation experiment has the following feature: since only one of the input phages has been irradiated, all of the functions necessary for phage production are represented in the infected cell; the survival curves for the contribution of genetic markers, then, whatever their basis, are not influenced by the failure of any of the three vulnerable centers to perform its function. We inquire into the origin of the marker survival curves by posing two questions: (1) How does ultraviolet light block the contribution of a genetic marker ("knock out" the marker)? (2) For those markers which are contributed, what is the mechanism by which the marker does appear in the progeny (is "rescued")? The work of Doermann and his collaborators has given satisfying answers to both of these questions.

*b. The Localized Nature of Ultraviolet-Induced Damage.* Doermann *et al.* (1955) executed a series of experiments well-suited to the detection of discrete lesions on the genetic structure of T4 if such were to exist. They employed as irradiated parent and reactivating parent, respectively, wild-type and triply mutant T4. The wild-type phage was adsorbed at low multiplicity (e.g., 0.2 particles per cell) to bacterial cells after various doses of ultraviolet light. The triple mutants were adsorbed simultaneously at a multiplicity just sufficient to infect almost all of the cells (e.g., 2-3 particles per cell). The infected cells were highly diluted and distributed into a large number of tubes such that only about 0.2 of the tubes received a cell within which a genetic contribution was made by an irradiated phage. After the cells had lysed, the contents of the tubes were assayed by the plaque assay method. Those plates which showed markers from the irradiated parent were then scored with respect to the eight possible genotypes of phage particles. All the data to be analyzed came from *within* this class of plates, so that conclusions drawn are based only on observation of irradiated phage which were competent to enter into the events leading to cross reactivation. The following conclusions obtain for this class of particles: (1) Markers which are unlinked in ordinary phage crosses (see Chapter VIII, Volume II) are independently "knocked out" by the ultraviolet irradiation. The markers employed showed approximately equal probability of knock out. If we define  $p$  as the probability that a given marker from an irradiated phage particle will appear in at least one copy in the progeny, then we can write the expected frequency of phage contributing 1, 2, or all 3 markers on the assumption of independent knockout. They are

$$\begin{aligned} P_1 &= p(1-p)^2/[1-(1-p)^3] \\ P_2 &= p^2(1-p)/[1-(1-p)^3] \\ P_3 &= p^3/[1-(1-p)^3]. \end{aligned} \tag{9}$$

At each dose, the data fit these expectations. (2) Markers which are linked tend to be knocked out together. The degree of correlation decreases with increasing map distance between the markers. (3) The knockout of one marker is correlated with a reduction in yield of a linked marker. Thus the ultraviolet hits which knockout markers are the same as those which cause a decrease in the number of copies in which a surviving marker appears.

These observations permit the conclusion that there exists a large class of ultraviolet hits which result in discrete lesions with localized effects on the genetic structure of the phage. A plot of  $\log p$  versus  $r$  gives a survival curve for a marker under conditions in which the survival is influenced only by these "genetic" hits (Fig. 5).

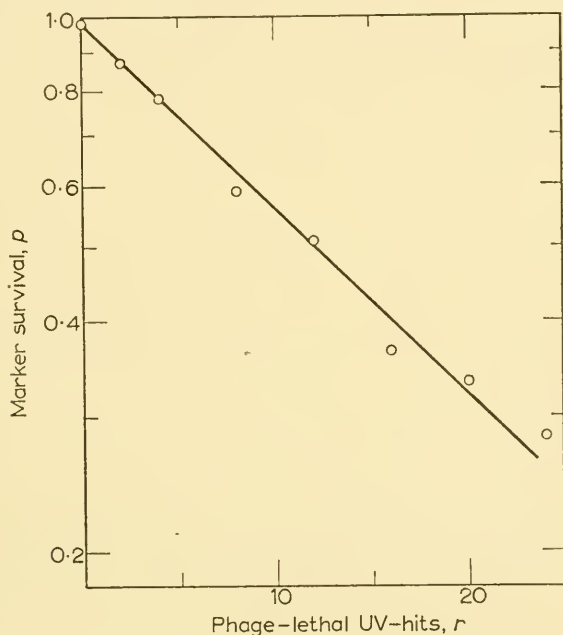


FIG. 5. The "knockout" at low doses of genetic markers in T4 by ultraviolet-induced lesions on the genetic structure. Among those particles which are able to participate in cross reactivation, 1 hit per 17 phage-lethal hits knocks out a given marker. The values for  $p$  are from Doermann *et al.* (1955) and have been corrected for certain systematic errors as described by Stahl (1956).

*c. The Mechanism of Cross Reactivation.* The above observations, together with the obvious observation that markers from the irradiated parent tend to appear in progeny particles which are genetic recombinants, tempt the conclusion that the mechanism of reactivation is the separation of a marker from the damages bracketing it by a process akin to, or identical to, the normal process of genetic recombination. Under this interpretation the



survival curve for a marker gives for each dose the probability that the marker will be recombined away from the nearest ultraviolet-induced damage on either side of it in the linear genetic structure. To examine the consequences of this notion we consider the simple case in which the points of recombination between the damaged and nonirradiated phage are not influenced by the position of the ultraviolet-induced lesions. Some phage in the irradiated suspension are destined by chance to have points of recombination only rather remote from the marker when they are subjected to the conditions of cross reactivation; others are destined to have points of recombination rather close to the marker. The marker in the first phage will be "sensitive"; that in the second phage will be relatively "resistant." The

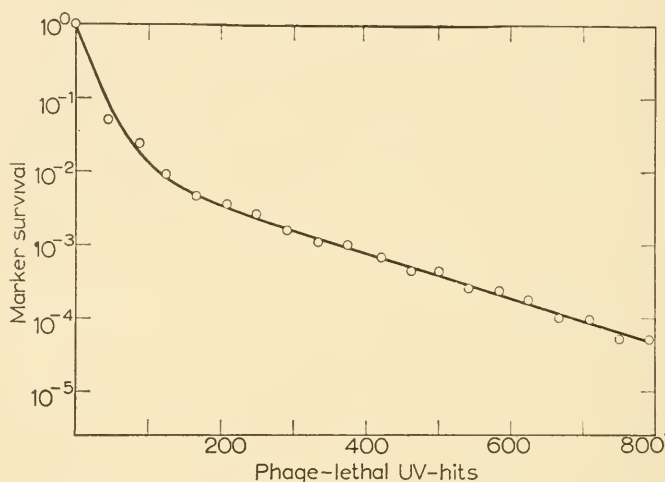


FIG. 6. Cross reactivation of a genetic marker of T4 at high doses of ultraviolet light. A culture of *Escherichia coli* B was infected with about two wild-type particles and two to three mutant particles. The fraction of cells liberating at least one wild-type particle at various doses of ultraviolet light administered to the wild-type parent was then determined. From this value, the fraction of parental irradiated particles contributing the wild-type marker was calculated for each dose. These values are plotted here logarithmically versus dose in phage-lethal hits. The figure was composed from unpublished data of Dr. A. H. Doermann and Mrs. M. Chase Epstein for the  $r_{47}^+$  marker of T4.

These data indicate the knockout of a marker as a result of *any* hits which influence the ability of the particle to make a genetic contribution. The close similarity in the low dose slope of this curve and the slope of the curve (Fig. 5) for marker survival influenced only by hits on the genetic structure indicates that few, if any, hits prevent a particle from participating in cross reactivation. The decrease in slope with increasing dose agrees with the notion that marker rescue occurs by recombination between an irradiated and a live phage. The sensitivity of the  $r_{47}^+$  marker at high dose is somewhat greater than the sensitivity of a number of other markers as determined in similar experiments. The difference in sensitivity is probably due to the fact that  $r_{47}$  is a "chromosomal aberration," whereas the other markers are "point mutations."

entire population may be thought of as a large number of subclasses within any one of which the given phage marker has a different sensitivity than in any other subclass. The basic philosophy of this idea is that for the most part a marker is knocked out not by a hit on it but by a hit near it; a prediction of this idea is that the survival curve for a marker should have a slope which decreases continuously with increasing dose of radiation.

Doermann and Chase (personal communication) have obtained survival curves for single markers up to doses of about 800 phage-lethal hits. Such a curve is shown in Fig. 6. The legend to the figure describes the technique by which the data were collected; here we wish only to indicate the essence of the method and point out several features of the survival curve. The technique measures the probability that an irradiated phage particle carrying a given marker will contribute the marker to at least one progeny particle in a cross-reactivation experiment. Any damage which reduces this probability in any way will contribute to the knockout of the marker, whether this damage is genic or nongenic. Two features of the curve argue for a very small upper limit to the amount of nongenic damage: (1) The final slope of the curve is less than 1 % as steep as the curve for the survival of infectivity; (2) the initial slope of the curve is almost the same as the initial slope of the curve determined under conditions which measure only genic damage (Fig. 5).

The progressively decreasing slope of the survival curve argues strongly for the recombinational nature of the marker reactivation. The significance of the apparently linear portion of the marker survival curve at high dose is quite in doubt. It has been variously interpreted as a measure of the minimum distance within which the two rescuing genetic exchanges can occur, a measure of the length of an ultraviolet-induced lesion (Barricelli, personal communication), or a measure of the sensitivity of a target upon whose survival the opportunity for cross reactivation of any part of the genome depends (i.e., a measure of the amount of nongenic damage).

Two modifications of this experiment lend support to the interpretation we have put upon it. In the first, wild-type phage is irradiated, and the reactivating phage is mutant for two closely linked markers. A reactivation is scored whenever at least one progeny particle contains both wild-type alleles. According to our interpretation, the low-dose survival curve in such an experiment should be approximately the same as that found when a single mutant phage is used as the reactivating parent. At high dose, however the survival curve should be steeper since any hit occurring *between* the pair of wild-type alleles would be expected to make reactivation of both markers into one undamaged vegetative phage essentially impossible. Furthermore, the slope should increase with increasing distance between the genetic markers employed. In the second experiment, the irradiated phage is mutant

for two closely linked markers; the reactivating phage is mutant for one marker which lies between the other two. A "reactivation" is scored whenever the wild-type allele at the central locus in the irradiated parent appears in a progeny phage particle along with wild-type alleles at each of the bracketing loci. Within our scheme, the mutant loci in the irradiated parent may be thought of as "ultraviolet-induced damages," which, even at zero dose, prevent reactivation in a fraction of the cases. In order for the reactivation to be rendered less likely by radiation, a damage must occur between the two mutant loci. Thus, the fraction of infected cells producing wild-type phage should fall below the curve obtained with a single marker until a dose is reached which assures that most of the phage have been hit between the two mutant loci. Then the curves should become identical. In both the above experiments, the predictions of the hypothesis of rescue by recombination are borne out in detail (Doermann and Chase, personal communication).

*d. Quantitative Aspects.* The evidence presented above indicates that some, at least, of the ultraviolet light-inactivation is due to hits on the genetic structure as we know it from mapping experiments (see Chapter VIII, Volume II). The same experiments permit estimates of the fraction of the total hits which fall into this category. One estimate can be derived from the initial slope of a cross-reactivation survival curve, another from the increment added to the final slope when two closely linked markers are used instead of a single marker.

To estimate the fraction of hits on the genome from an examination of the initial portion of a marker survival curve, we need values for: (1) the slope of the curve; (2) the map distance between two markers which are just far enough apart to be essentially independently knocked out at low dose; and (3) the total map length of the T4 genome. The experiments of Doermann *et al.* give the first two values as 0.06 and 12-20 (we shall use 16), respectively. The total map length is not accurately known but is probably at least 200 units. The total genome can accommodate  $200/16 = 13$  independently inactivated regions. Since each of these regions has a sensitivity of about 0.06, we conclude that  $0.06 \times 13 = 0.8$  of the ultraviolet hits are accounted for by low-dose cross-reactivation experiments.

To estimate the fraction of hits on the genome from high-dose data, we need values for: (1) the increase in ultimate slope arising from the requirement of a two-marker reactivation, along with (2) the map distance between the two factors, and (3) the total map length. For the marker pair,  $r_{205}-r_{320}$ , for instance, the first two values are 0.0021 and 0.47, respectively. Thus, ultraviolet light delivers  $0.0021/0.47 = 0.0045$  hits per map unit per phage-lethal hit. Again assuming a map length of 200 units, we calculate that  $0.0045 \times 200 = 0.9$  of the ultraviolet hits to the phage are accounted for by the high-dose experiments.

The conclusion from the high- and low-dose cross-reactivation experiments are in eminent accord with our working hypothesis for the basis of multiplicity reactivation.

#### 4. Inactivation of Genic Function

Mutants of T4, called *rII*, cannot grow in *Escherichia coli* K12 ( $\lambda$ ), (Benzer, 1955). Wild-type T4 grows well in K12( $\lambda$ ). The *rII* mutants fall into two groups, called A and B; a simultaneous infection of K12( $\lambda$ ) by a mutant of group A and one of group B leads to the apparently normal production of phage; a mixed infection in which both mutant phages come from the same group fails to yield phage. Mutants of group A all occur within one region of the genome about 5 map units long; those in group B all occur within a region about 3 map units long. The two regions, called

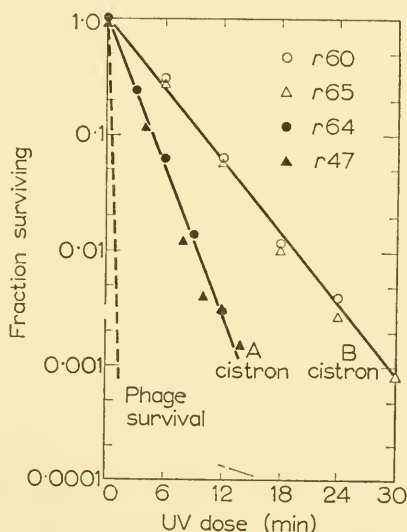


FIG. 7. The survival of the function of *rII* cistrons in T4 following ultraviolet irradiation. The slopes of the survival curves indicate that the functions of the A and B cistrons are inactivated by 0.1 and 0.05 of the phage-lethal hits, respectively. The figure is adapted from Krieg (1957).

“cistrons” (Benzer, 1957), are adjacent on the genetic map. We may define the function of the wild-type state of an *rII* cistron as its power in mixed infection to permit particles which are mutant at that cistron to grow in K12( $\lambda$ ). Krieg (1958) has studied the inactivation by ultraviolet light of this genic function. He found that 0.1 of the phage lethal hits inactivate the function of the A cistron; the B cistron is inactivated by 0.05 of the total phage-lethal hits. The survival curves for each cistron suggest strongly that one hit is sufficient to inactivate the function of a cistron (Fig. 7). With



regard to our notion about the nature of multiplicity reactivation, these results are encouraging in two respects: (1) They indicate that a phage particle inactivated by ultraviolet light can perform a function on behalf of other particles within the cell. (2) The function of a cistron can be inactivated by ultraviolet light at a rate approximately that expected for a vulnerable center, i.e.,  $0.4/3$  of the rate of phage inactivation.

An extension of the above experiments show that hits which inactivate the function of the *rII* cistrons are (at least in part) identical with those which knock out a marker in cross-reactivation experiments. Among those cells which liberated phage in the function-inactivation experiments, Krieg (1958) scored for the cross reactivation of the *r<sup>+</sup>* marker. He found that the probability of cross reactivation among these cells became constant at a value of 0.14 (for markers in the B cistron). The knockout of a remote marker in the same phage proceeded to much lower values. This experiment, therefore, in automatically selecting for phage with intact B-cistron function, simultaneously selects for particles which are free of hits in the immediate vicinity of the marker but have suffered typically in other regions of the genome. Thus, the *rII* cistrons themselves (as opposed, for instance, to some product of the *rII* cistrons brought in by the infecting phage) must be hit to inactivate the function of the wild-type allele.

Other experiments by Krieg (personal communication) show that if an *rII* wild-type cistron is to function at all in determining growth in K12( $\lambda$ ), it must be present in the input phage; i.e., there is no opportunity for it to form by genetic recombination and *then* express its function. Thus the *rII* cistrons have many of the properties expected of "vulnerable centers." Since *rII* mutants, and even chromosomal aberrations which may be deletions (Benzer, 1955) involving the *rII* region grow well in *E. coli* B (the commonly employed host cell), the *rII* cistrons almost certainly do not correspond per se to the vulnerable centers revealed by standard multiplicity reactivation experiments. However, they should reveal themselves in multiplicity reactivation experiments which use K12( $\lambda$ ) as host cells. This expectation is borne out by the experiments described below.

### 5. Multiplicity Reactivation of T4 in Cells of *Escherichia coli* K12( $\lambda$ )

*a. The rII Cistrons as Vulnerable Centers.* Krieg demonstrated that a wild-type *rII* cistron in an irradiated particle could perform its function on behalf of an unirradiated *rII*-mutant particle in the same cell. To qualify fully as vulnerable centers, the *rII* cistrons in irradiated phage must be shown to be able to perform functions which lead to the production of phage in the presence of only other *irradiated* phage. Epstein (1958) has demonstrated that the *rII* cistrons do behave in this fashion. He compared multiplicity reactivation in the following two experiments: (1) Wild-type T4 was irradiated and

tested for multiplicity reactivation in K12( $\lambda$ ); (2) a mixture of an *r*IIA mutant stock and an *r*IIB mutant stock was irradiated and tested for multiplicity reactivation in the same fashion. The only difference between the two experiments is that in Experiment (2) a smaller number of wild-type *r*II cistrons is introduced into each cell. It was found that at all doses tested (up to 9 hits per phage), the survival of the wild-type multicompleses was higher than the survival of the mixed *r*II multicompleses.

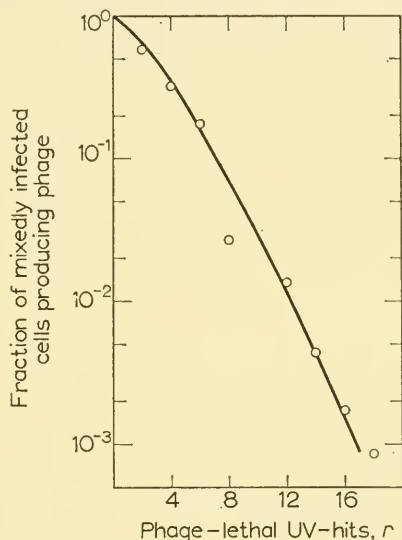


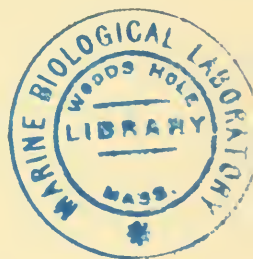
FIG. 8. Multiplicity reactivation of ultraviolet-irradiated T4 in *Escherichia coli* K12( $\lambda$ ). A mixture of equal numbers of *r*IIA and *r*IIB particles was irradiated and adsorbed at low (0.24 particles per cell) multiplicity on K12( $\lambda$ ). The smooth curve is the sum of the theoretical survival curves for bi- and tricompleses weighed according to their relative frequency among productive cells at zero dose. On the assumption that *r*II cistrons act as vulnerable centers in K12( $\lambda$ ), the survivals of the bi- and tricompleses, respectively, are given by

$$y_2 = [1 - (1 - e^{-fr/n})^2]^n e^{-0.15r}$$

$$y_3 = [1 - (1 - e^{-fr/n})^3]^n e^{-0.075r} [1 - (1 - e^{-0.075r})^2]$$

From Harm (1956) (see Fig. 4), we estimate *f* (the fraction of hits on vulnerable centers) to be 0.4 and *n* to be 3. The number 0.15 is the fraction of hits on the *r*II cistrons (see Fig. 7). The calculation of *y*<sub>3</sub> was simplified by assuming that the two cistrons were of equal sensitivity. For a total multiplicity of 0.24, the productive complexes at zero dose have frequencies of 0.86 and 0.14, respectively, for the bi- and tricompleses. The data are from unpublished experiments of Dr. R. H. Epstein.

One experiment was performed under conditions which makes it possible to test quantitatively (although not very critically) the notion that the *r*II cistrons act as an additional pair of vulnerable centers when multiplicity



reaction is measured in K12( $\lambda$ ). In mixed infection of K12( $\lambda$ ) by an *r*IIA and an *r*IIB mutant, only those cells which are infected by at least one of each type of mutant produce phage. If the two types of phage are adsorbed to K12( $\lambda$ ) at low (cir. 0.1) multiplicity of each type, the majority of cells producing phage do so by virtue of having been infected by exactly one of each type; a calculable number contain two of one type and one of the other; an insignificant proportion contains two of each type. The theoretical survivals for the mixed doubly and triply infected cells are obtained by simple modification of Equation (5). We assume that the average sensitivity for the function of an *r*II cistron is 0.075 of the total sensitivity and that the three vulnerable centers which act in *E. coli* B as well as in K12( $\lambda$ ) comprise 0.40 of the total target. Figure 8 compares Epstein's (personal communication) data with the curve generated by these considerations with encouraging results.

*b. Genetic Recombination During Multiplicity Reactivation.* In addition to being of different genotype in the *r*II region, the input phage in a low-

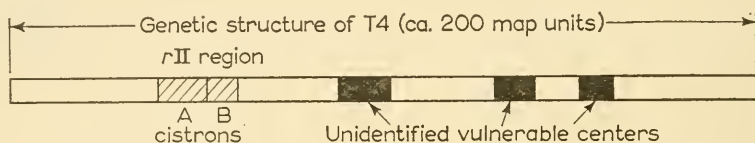


FIG. 9. The structure of T4 as revealed by studies on ultraviolet-inactivated phage. The primary target for ultraviolet light is the linear genetic structure. Three regions (shaded) of the genome perform functions necessary for duplication and recombination. The A and B cistrons of the *r*II region (cross-hatched) have such properties when T4 is examined in the host K12( $\lambda$ ).

multiplicity experiment in K12( $\lambda$ ) may be made different at other loci whose functional survival does not determine the ability to multiply. In these experiments, the frequency of phage recombinant for these nonselective markers increases with dose of ultraviolet light delivered to the parents (Epstein, 1958). Furthermore, at high doses, a number of yields from single bacterial cells are composed over 90 % of one genotype which is often one of the recombinant genotypes. This is the result demanded by the hypothesis that recombination between the input phages reconstitutes a genome free of damage which then multiplies to become the predominant type in the cell. The other genotypes released in small numbers in any given cell arise by cross-reacting interactions between this viable multiplying population and the inactive particles in the cell.

On the basis of the experiments described, we can construct a formal picture of T4 as it appears to a photon of ultraviolet light (Fig. 9).

## *B. Comparative Radiobiology of T2 and T4*

### *1. Photoreactivation*

*a. Photoreactivable Sector.* It was observed by Dulbecco (1949) that the infectivity of a fraction of the inactive particles in an irradiated phage suspension could be restored by shining visible light on cells to which the inactive phage had adsorbed. The degree to which photoreactivation can occur differs among the T-phages; of the T-even phages, T2 shows the greatest degree of photoreactivation and has been the principal object of study. A survival curve for infectivity under conditions of maximal photoreactivation can be obtained. Such a curve is essentially identical to the survival curve obtained without photoreactivation, except that the slope is reduced. The fraction by which the slope is reduced is a characteristic of the phage and is referred to as the "photoreactivable sector." The photoreactivable sector for T2 is 0.56 and for T4 is 0.2 (Dulbecco, 1950).

Photoreactivation could be interpreted in two general ways; within these interpretations a variety of specific schemes is possible. (1) Visible light performs some function which in the dark must be carried out by the phage particle. (2) Visible light erases a fraction of the hits in the phage population. As pointed out by Bowen (1953), the widespread occurrence of photoreactivation makes the second interpretation far more likely.

An experiment by Lennox *et al.* (1954) is strongly in support of the idea of erasure of ultraviolet-induced damage. They irradiated two different populations of infected cells and measured their ability to produce phage. One population was irradiated and then photoreactivated; the other population had been irradiated to the survival level of the first population following its photoreactivation. The sensitivities of these populations to additional ultraviolet irradiation was found to be identical.

*b. Kinetics of Photoreactivation.* The restoration of infectivity as a function of dose of reactivating light has been examined by Dulbecco (1950 and 1955) and Bowen (1952). With phage T3, reactivation shows the expected "multi-hit" kinetics; the number of quanta required to restore infectivity to a reactivable particle is equal to the number of hits which the particle has received (Dulbecco, 1955). For T2, however, the results are puzzling. The T2 preparations used by Bowen had received on the average about 7 hits. Since the photoreactivable sector for T2 is 0.6, about 4 hits per particle must be thought of as being "reactivable." He found, however, that to a good approximation the restoration of infectivity responds to visible light *as if* only one quantum of light were required to reactivate a given phage particle. Bowen has indicated (1952) how this finding might be reconciled with the idea of erasure of damage. He suggests that reactivable hits are of several classes. The frequency and quantum efficiency for the restoration of each



of these classes is such that the kinetics of reactivation look one-hit, although an average of 4 quanta per phage are required. Although this explanation is strictly *a posteriori*, the even greater unpleasantness of possible alternative explanations encourages tolerance of Bowen's suggestion.

## 2. Genetic Control of Sensitivity to Ultraviolet Light

The sensitivities of T2 and T4 differ by a factor of about two. Streisinger (1956) has shown that this difference is inherited in crosses as if it were controlled by a single genetic factor, the *u* gene. The *u* allele, which confers resistance, may be supposed to act in one of the following ways: (1) It may render the DNA of the particle intrinsically less sensitive by (a) lowering the absorption coefficient for ultraviolet light, or (b) by lowering the quantum efficiency for the production of lethal damages by adsorbed photons. (2) It may free the phage from dependence on a set of sensitive parts of the phage by rendering these parts superfluous. (3) It may be able to erase about one-half of the ultraviolet-induced lesions.

Streisinger (1956) ruled out possibility (1a) by demonstrating that the absorption of ultraviolet light was the same for *u* and *u*<sup>+</sup> phage. The possibilities (1a) and (1b) are simultaneously eliminated by the following experiment by Harm (1958b): He obtained survival curves for irradiated *u*<sup>+</sup> phage assayed under three different conditions: (I) on ordinary *E. coli* B, (II) on *E. coli* B infected with several particles per cell of heavily irradiated (cir. 140 hits) *u*<sup>+</sup> phage, and (III) on *E. coli* B infected with several particles per cell of heavily irradiated *u* phage. The survival curve in the first two cases was that typical of *u*<sup>+</sup> phage; in the last case the curve obtained was characteristic of *u* phage.

Harm (personal communication) found that the effects of photoreactivating light and heavily irradiated *u* phage on the reduction in sensitivity of *u*<sup>+</sup> phage is much less than additive. The simplest explanation seems to be that photoreactivating light and the *u* gene work by erasing classes of damages which are in large part common. A similarity in action of photoreactivation and the *u* allele is consistent with X-ray and suicide experiments. With both these agents, T2 and T4 have very similar sensitivities, and very slight photoreactivation, if any, can be observed.

A detailed comparison of the radiobiology of *u* and *u*<sup>+</sup> phage should prove rewarding on two counts. It could lead to an understanding of the mechanism of action of this interesting gene, and it could further restrict our picture of T-even phage as deduced from irradiation experiments. To date, the following differences have been established for *u* and *u*<sup>+</sup> phage (either by comparison of T2 and T4 or by comparison of T2*u*<sup>+</sup> and T2 into which the *u* gene has been crossed from T4): (1) The plaque-forming ability for T2 is about twice as sensitive as that for T4. (2) The photoreactivable sector is

about 0.6 for T2 and 0.2 for T4. (3) In multiplicity reactivation experiments, the slope at high dose is about 0.2 that of whole phage for T2 and 0.4 for T4.<sup>1</sup> (4) In cross-reactivation experiments, with T2u<sup>+</sup> phage as the un-irradiated parent, the initial slope for marker knockout (log survival versus minutes of irradiation) is about twice as steep for T2u<sup>+</sup> as for T2u, whereas at higher doses the curves are identical for the two phages (Harm, personal communication). (5) In intracellular irradiation experiments (see Section C1b) the sensitivity of T4 (or T2u) does not change for the first 5 to 6 minutes of the infectious cycle, whereas the sensitivity of T2u decreases almost two-fold during this period (Streisinger, 1956; Symonds and McCloy, 1958). This change in sensitivity of T2u<sup>+</sup> disappears under conditions of maximum photoreactivation (Symonds and McCloy, 1958).

We offer these observations without comment except to say that they do not all fit easily into our radiological model for T4.

### C. Irradiation of Infected Cells

#### 1. Luria-Latarjet Experiment

a. *Purpose of the Experiment and Results with T7.* In some phage systems, the ability of the host cell to produce phage upon subsequent infection is not appreciably impaired by irradiation (e.g., see Anderson, 1948). In such cases, moreover, the ability of the cell to produce phage is inactivated at approximately the same rate as are free phage particles if the irradiation is given to singly infected cells immediately following infection. This situation makes it possible to study the evolution of the infectious process by determining the changes in sensitivity of the infected cells (Luria and Latarjet, 1947). This potentiality is best illustrated by a discussion of a simple case.

One may imagine that the only changes in sensitivity which occur arise as a result of multiplication of the infecting phage. In the absence of interactions between the new particles (i.e., in the absence of multiplicity reactivation), the sensitivity of the complexes would change in a very simple way;

<sup>1</sup> The significance of this comparison between T2 and T4 is somewhat doubtful. Harm, working with T4 (1956), assumed that the ability of phage to participate in multiplicity reactivation was independent of dose; Dulbecco, working with T2 (1952a), assumed that particles which had lost the ability to kill bacteria upon single infection had lost the ability to participate in multiplicity reactivation. Each calculated  $x$ , the multiplicity of infection, accordingly.

In T2, one hit per 120 inactivates bacterial killing ability (Dulbecco, 1952b), whereas in T4 one hit per 50 is effective (Doermann *et al.*, 1955). Thus, if Harm's assumption is the correct one, the final slopes for T2 and T4 retain most of their difference. If Dulbecco's assumption is correct, however, the difference in final slope for T2 and T4 may be very small. We have seen that the rate at which ultraviolet light destroys the ability of T4 to participate in *cross reactivation* is very small. This suggests that Harm's assumption is the correct one.

the survival curves would become progressively "multihit," gaining a shoulder whose extent increased with the increase in the number of intracellular particles. Such an ideal result is found for the phage T7 (Fig. 10). For the T-even phages, however, the findings are not so easily interpretable.

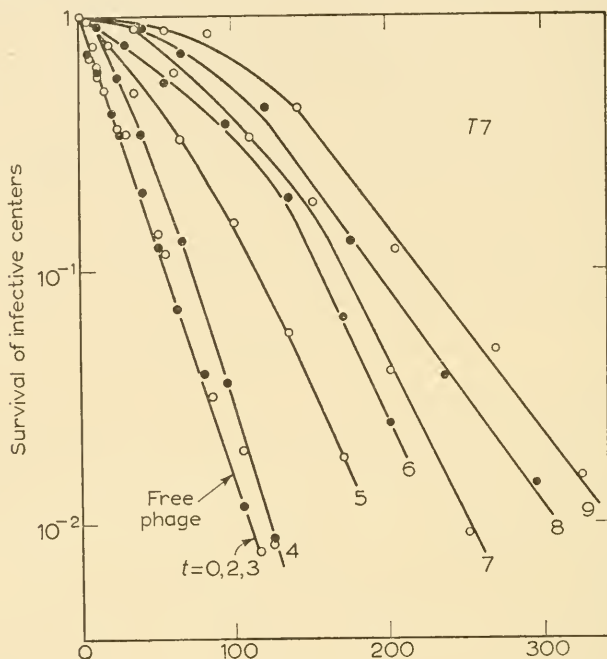


FIG. 10. Survival curves for singly infected T7 complexes irradiated with ultraviolet light at different times during the latent period. Each curve is marked with the time in minutes following infection. The latent period for T7 is 12 minutes long. This figure is from Benzer (1952) and is reproduced by permission of the Williams and Wilkins Company.

*b. Results with T-even Phage.* Immediately following infection, the ability of a cell infected singly with T2 or T4 to produce a plaque is lost at about the same rate as is the plaque-forming ability of free phage (Luria and Latarjet, 1947; Benzer, 1952). The changes in sensitivity thereafter are best seen in the precise survival curves obtained by Benzer (1952) with T2, which are depicted in Fig. 11. It may be seen that between 0 and 6 minutes the sensitivity decreases by about a factor of two. Thereafter, there is a large and rapid decrease in sensitivity to a value less than one-tenth the initial value. In order to guide the discussion, we adopt the following hypothesis, which is a direct consequence of our views on multiplicity reactivation:

During the first 6 minutes, the phage performs those functions which are required to initiate the synthesis of vegetative phage. On the simplest model,

this period should be marked by no change in sensitivity. Such a result is found for T4 growing in *E. coli* B (Streisinger, 1956; Symonds and McCloy, 1958). The idea (invoked by Krieg (1958) to explain the high sensitivity of the function of the  $rII$  cistrons) of two classes of damage, both of which inactivate function, but one of which can be erased by the act of duplication, might explain the slight change observed with T2 (Symonds and McCloy 1958).

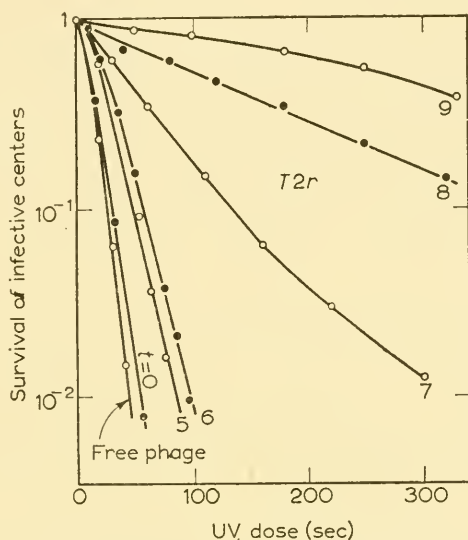


FIG. 11. Survival curves for singly infected T2 complexes irradiated with ultraviolet light at different times during the latent period. Each curve is marked with the time in minutes following infection. The latent period for T2 is 19 minutes long. This figure is from Benzer (1952) and is reproduced by permission of the Williams and Wilkins Company.

The rapid change in sensitivity after 6 minutes marks the formation of the first new vegetative particles and, thereby, the opportunity for multiplicity reactivation. Thereafter, since all preduplicational functions have been performed, multiplicity reactivation occurs with essentially 100 % efficiency rendering the complexes highly resistant.

The residual sensitivity at the time of maximal resistance may reflect: (1) a slight dependence of multiplicity reactivation on nonvulnerable parts of the genome (Barricelli, 1956); (2) the dependence of the complex upon a few phage-producing centers which have a slight but significant sensitivity (Epstein, H. T., 1956); or (3) the loss of complexes due to lysis of the cells before the completion of the reactivation (Symonds, 1957).

The interpretation given here to the Luria-Latarjet experiment with T-even phage finds support in four observations. (1) DNA synthesis begins



about 6 minutes after infection (see Chapter VII, Volume II). (2) This DNA synthesis must be preceded by protein synthesis (see Chapter VII, Volume II). (3) The function of the *rII* cistrons becomes insensitive to suicide during the first 6 minutes following infection (Steinberg, personal communication). This point will be expanded in Section III. (4) Recombination is stimulated by irradiation of stable (10-minute) complexes. This point is discussed below.

## 2. *Effects of Ultraviolet Light on Vegetative Phage*

Tomizawa (1958) irradiated infected cells after the attainment of resistance and then examined the emerging progeny phage. At sufficiently high dose, all the emerging progeny were unable to form plaques. In multiplicity, cross, and photoreactivation experiments they showed the response characteristic of a population of phages which had been irradiated in the free state with the same dose of ultraviolet light.

Hershey (1958) irradiated cells infected with two genotypes of phage after the onset of DNA production. The fraction of recombinants in the progeny increases with increasing dose. Furthermore, the yields from such cells at high doses tend to be genotypically pure (Hershey, personal communication). As we have seen, this is exactly the genetic consequence of multiplicity reactivation when the irradiation is given prior to infection (Epstein, 1958).

# III. THE INACTIVATION OF T-EVEN PHAGE BY THE DECAY OF INCORPORATED $P^{32}$ (SUICIDE)

## A. *Basic Procedures*

### 1. *Preparation of Radioactive Phage*

Phage containing large amounts of  $P^{32}$  are prepared by infecting with a small number of phage particles a culture of cells which has been growing in medium of high specific activity. The inoculum of phage is usually so adjusted that after two cycles of infection the great majority of the cells have lysed. The phage are then freed if necessary from the radioactive culture medium and cell debris and stored at sufficiently low concentration that the beta ray activity has no significant effect on the viability of the particles.

### 2. *Survival Curves*

The  $P^{32}$  within the DNA of the phage particles decays according to the equation

$$N = N_0 e^{-\lambda t} \quad (10)$$

where  $\lambda$  is the decay constant of the isotope,  $N_0$  is the original number of  $P^{32}$  atoms, and  $N$  is the number remaining at time  $t$ . For  $t$  measured in days,  $\lambda$  is about 0.05.

For  $\alpha =$  to the quantum efficiency (the probability that a decay will cause the inactivation of a phage particle), we see that the average number of hits at time  $t$  is

$$r = \alpha N_o(1 - e^{-\lambda t}) \quad (11)$$

$N_o$  is limited by the requirement that the medium have sufficiently low specific activity to support the growth of bacteria. Therefore, most experiments must be carried out over a length of time which permits the decay of the majority of the incorporated atoms. A survival curve is generated by assays of the stored radioactive phage stock at approximately daily intervals over a period of 2 to 4 weeks. The survival curve is an exponential function of  $r$  [Equation (3)] (Hershey *et al.*, 1951; Stent and Fuerst, 1955). The suicide of phage following infection is measured by periodic assay of infected cells maintained in liquid nitrogen (Stent, 1953a).

### *B. Cross and Multiplicity Reactivation*

Cross reactivation experiments with suiciding phage have been performed with intracellularly inactivated T2 (Stent, 1953a) and with extracellularly inactivated T4 (Stahl, 1956). The intracellular experiments suffer somewhat from fluctuations in the properties of the thawed samples; the extracellular experiments are complicated by a dose-dependent loss of the ability of suiciding phage to inject DNA into the host cell (Tomizawa, personal communication). The two techniques give results which lead to essentially the same conclusions. As with ultraviolet light, linked markers are knocked out (by genic hits) with a correlation that depends on the map distance between them (Stahl, 1956). Unlinked markers are inactivated by genic hits independently (Stent, 1953a) or nearly so (Stahl, 1956). However, crossreactivation of markers from suicide phage differs from the results with ultraviolet irradiated phage in two respects: (1) A given phage-lethal  $P^{32}$  hit has a probability of knocking out a marker which is several times that found with ultraviolet light. (2) The burst size of reactivated markers is only slightly less than the burst size of the marker in the zero-dose sample; with ultraviolet light, the burst size of the reactivated marker is depressed to a value of between 1 and 2 at high dose (Doermann *et al.*, 1955).

In view of the reduction in ability of suicide phage to inject DNA, it is not surprising that multiplicity reactivation has not been demonstrated for phage which has been permitted to suicide extracellularly. It seems somewhat surprising, however, that phage permitted to decay inside the cell also fails to give multiplicity reactivation (Stent and Fuerst, 1955).

*C. Luria-Latarjet Experiments*

In these experiments, cells infected singly with radioactive phage are allowed to incubate for various lengths of time before their development is suspended by freezing. With T2, the survival curves obtained by periodic assay of these frozen samples are highly reminiscent of the curves obtained with ultraviolet light. For the first 5 to 6 minutes there is very little (Stent, 1955) or no (Symonds and McCloy, 1958) change in sensitivity of the complexes. Thereafter, there is a rapid rise of resistance, so that by about 9 minutes the complexes are apparently totally resistant to the decay of phosphorus in the infecting particle.

The obvious interpretation of this experiment is that by 9 minutes there is in every cell at least one new, nonradioactive copy of the infecting phage. This would insure phage production by these cells independently of the inactivation of the original, radioactive, infecting particle (Stent, 1953b). This simple interpretation was simply ruled out by a repeat of the same experiment using *radioactive* bacteria growing in radioactive medium (Stent, 1955). The complexes stabilized in essentially the same fashion. Stent (1955) suggested three interpretations of this result: (1) Phage DNA in the cells at 9 minutes is in a  $P^{32}$ -resistant state. (2) The genetic information of the infecting particle has been transferred to a  $P^{32}$ -resistant structure. (3) Multiplicity reactivation at 9 minutes is highly efficient. The following experiment (Steinberg, personal communication) suggests that multiplicity reactivation, although not demonstrable for phages which are allowed to decay at an early stage in development, might indeed be efficient for phages inactivated after 9 minutes of development.

Steinberg infected K12( $\lambda$ ) with radioactive wild-type T4 at a low (ca. 0.1 particles per cell) multiplicity and with about 1 per cell of a T4rII mutant which was not radioactive. The infected cells were allowed to develop for various lengths of time before they were frozen. Survival curves for the function of the rII cistron were then obtained by assaying at intervals for the ability of the cells to yield phage. Immediately following infection, the function of the rII cistron was about one-third as sensitive as the whole phage. By 6 minutes, however, the function had become so resistant that no inactivation was detected. This finding suggests that whereas multiplicity reactivation might be inefficient in very early complexes because of the extreme sensitivity of the function of cistrons required for duplication, it might be thoroughly efficient after 6 minutes, by which time these functions have been performed.

At present it seems that the Luria-Latarjet experiments with suicided phage may well have an explanation essentially identical to that proposed for ultraviolet-irradiated complexes. In this regard, it would be helpful to know the effect of  $P^{32}$  decay on genetic recombination in stable, 9-minute complexes.

## IV. X-RAY INACTIVATION OF T-EVEN PHAGE

*A. Survival of Infectivity*

X-rays applied either intracellularly or extracellularly produce exponential inactivation (Fig. 1) under conditions in which indirect effects are avoided (see Chapter V, Volume I). Whereas with both ultraviolet light and suicide the phage immediately following infection is slightly less sensitive than free phage (Benzer, 1952; Stent, 1955), with gamma rays the sensitivity is the same in both cases (Harm, 1958a), and with soft X-rays, the infected cells are *more* sensitive than the free phage (Harm, 1958a; Symonds and McCloy, 1958). The equality in sensitivity with gamma rays indicates that essentially all of the damage produced by ionizing radiations is damage to the DNA of the phage, and that the biological effectiveness of an individual ionization does not change as a consequence of injection. The increase in sensitivity to soft X-rays following injection of the DNA must, then, reflect a relatively "inefficient" *distribution* of hits among phages irradiated as free particles with soft X-rays. This inefficiency results from the occasional production by one photoelectron of two or more effective ionizations within the same particle. (The change to a more efficient distribution upon injection reflects a change in shape of the DNA mass of the phage from a compact form to an elongated or flattened form (Harm, 1958a).) It follows that at any given survival level, the average number of hits per inactive particle among free phages irradiated with soft X-rays is greater than is the case with gamma rays or with phage irradiated after injection. This may in part account for the differences in behavior (see below) of phage inactivated with soft or hard X-rays, or with soft X-rays applied before or after infection.

*B. Multiplicity and Cross Reactivation*

For radiation applied to free phage, the degree of multiplicity reactivation is slight and increases with decreasing ionization density (Weigle and Bertani, 1956). The dependence on ionization density is probably a simple consequence of the bunching of hits described above (Harm, 1958a).

When X-rays are applied to phage-infected cells, multiplicity reactivation occurs with an efficiency comparable to that found with ultraviolet light (Weigle and Bertani, 1956; Harm, 1958a). Cross reactivation, which is rather inefficient when X-rays are applied to free phage (Doermann and Chase, reported by Stahl, 1956; Harm, 1958a) is also highly efficient when the radiation is applied to cells following infection (Harm, 1958a). It seems likely that when ionizing radiation is applied to free phage a dose-dependent



fraction of the genetic material of the population is prevented from entering into the acts leading to multiplicity or cross reactivation (Weigle and Bertani, 1956; Harm, 1958a). This probably reflects a loss of ability of such phage to inject its DNA (Hershey *et al.*, 1954).

Genetic recombination increases when X-rays are applied to infected cells (Harm, 1958a). Qualitatively, multiplicity reactivation with X-rays applied to infected cells looks similar indeed to multiplicity reactivation with ultraviolet light. A quantitative comparison is presently not profitable since, necessarily, the X-ray experiments involve delivering to the host cell the same dose of X-rays which one delivers to the phage. It has been shown (Weigle and Bertani, 1956; Harm, 1958a) that the efficiency of multiplicity reactivation with X-rayed free phage is increased by X-ray treatment of the host cells. Indeed, Harm has shown that X-rays delivered to the host cells increases the cross reactivation of ultraviolet-inactivated phage! A quantitative comparison of multiplicity reactivation with ultraviolet light and X-rays applied to infected cells must await a systematic study of the effects of X-rays delivered to the host cell on the outcome of multiplicity reactivation experiments with ultraviolet-inactivated phage.

### *C. Luria-Latarjet Experiments*

Luria-Latarjet experiments performed with X-rays show similarities with the analogous experiments performed with ultraviolet light and P<sup>32</sup>. As with P<sup>32</sup>, X-rays delivered to T2-infected cells fail to reveal the slight change in sensitivity characteristic of T2 irradiated with ultraviolet light during the first 5 to 6 minutes (Symonds and McCloy, 1958). Thereafter, the sensitivity of the complexes decreases rapidly, but the complexes never achieve the high degree of resistance found with P<sup>32</sup> and ultraviolet light (Latarjet, 1948; Stent, 1955). Instead they become "multihit" with a target number around 10-20 (Latarjet, 1948) and a slope at high dose which is about one-half that characteristic of the complexes irradiated immediately following infection.

At present, the similarities between the Luria-Latarjet experiments with the three agents appear more significant than their differences. All may be interpretable on the same basis. As with multiplicity reactivation, more careful comparative studies are needed to ascertain whether the differences which do exist reflect anything more significant than differing side effects on the host cells.

## V. A SURVEY OF OTHER PHAGES

### *A. Introductory Remarks*

The T-even phages stand in contrast to most of the other known phages in the degree to which their development is independent of the nuclear

apparatus of the host cell. As a consequence, the ideas, if not always the experiments, on their radiobiology are simpler, and form a natural unit for the purposes of this review. We have exploited their exceptional behavior in order to introduce the principal concepts and basic techniques of phage radiobiology. Armed with these concepts, we may now look elsewhere.

### *B. Survival of Infectivity*

The inactivation of all phages by ionizing radiations (direct effect) or by  $P^{32}$ -suicide is exponential. The sensitivities to these agents are in general in approximate proportion to their DNA content (Epstein, 1953; Stent and Fuerst, 1955). The small phages S13 and  $\phi$ X174 are anomalously sensitive to suicide (Tessman *et al.*, 1957; Tessman, personal communication). It seems most likely that this is causally related to the aberrant physical properties of the DNA of this phage (Sinsheimer, personal communication).

In contrast with the simple survival curves obtained with ionizing radiations or  $P^{32}$ -suicide, the curves found with ultraviolet light assume every imaginable shape. The suggestion (Garen and Zinder, 1955) that such curves often reflect a partial reactivation of the phage by the host cell seems substantiated for a number of systems (e.g., for *Salmonella* phage P22 and *E. coli* phage T3 (Garen and Zinder, 1955), and for T1 (Garen and Zinder, 1955; Tessman and Ozaki, 1957). These experiments in general consist of a demonstration that the survival curves for phage assayed on irradiated host cells is steeper than when the assay is made on normal cells. At intermediate doses to the host cells, the ultraviolet-survival curves of the phage are two-limbed. The contribution of the high-dose limb to the survival of the phage at zero dose represents the fraction of cells in which the reactivating capacity remains intact.

It is not clear whether the mechanism of reactivation of the inactive phage by the host cell is by erasure or replacement, perhaps as a result of genetic recombination between the phage and homologous structures in the host cells. The ultraviolet-induction of mutations (see Section V, D) in many such systems, however, argues for a recombinational mechanism of reactivation. Arguing against a recombinational mechanism are the observations that with X-ray or suicide-inactivated phages cellular reactivation does not appear to occur. Perhaps if these agents were to be delivered to the phage after infection, host-cell reactivation might occur in analogy with the increased efficiency of cross reactivation of T-even phage under these conditions.

### *C. Multiplicity and Cross Reactivation*

In those systems showing host-cell reactivation, multiplicity and cross reactivation are relatively difficult to demonstrate. Tessman and Ozaki

(1957) showed that this can be the direct consequence of host-cell reactivation. They found a considerable increase in multiplicity reactivation of T1 on cells which had lost reactivating power as a result of ultraviolet irradiation.

Two not mutually exclusive explanations for the small amount of multiplicity or cross reactivation in systems showing host-cell reactivation can be offered: (1) The reactivation mechanism works in such a way that a fraction of the particles are completely reactivated while the number of hits on the remaining particles is not reduced at all. (A recombinational mechanism of reactivation might be expected to have this consequence.) As a result, the inactive particles in an irradiated phage stock have a higher number of hits than would be calculated from the fraction of survivors assuming a Poisson distribution of hits. They would, therefore, show an apparently reduced ability to be cross or multiplicity reactivated relative to T-even phage. (2) Vegetative phages in systems showing host-cell reactivation tend to mate with their homolog in the host cell rather than with each other. This would lead to a reduction in the amount of phage-phage reactivation, which depends, as we have seen, on recombination between the phages. In line with this view is the observation that with such phages the number of rounds of mating in normal crosses is less than with the T-even phages (see Stent, 1958, for an extended discussion of this point).

#### *D. The Induction of Mutations by Radiation*

There has been no report of an increased mutation frequency in T-even phages among the survivors of ultraviolet irradiation. However, increased mutation frequencies have been observed for phage produced by multiplicity reactivation following ultraviolet irradiation. Latarjet (1949, 1954) irradiated T2-infected cells after the onset of vegetative phage multiplication (after 6 minutes). The fraction of cells surviving the irradiation and liberating host-range mutants increased. Krieg (personal communication) has observed reversions to wild-type of T4 *r*II mutants. He applied the ultraviolet light to free phage and observed the progeny produced following multiple infection. It remains to be decided whether the role of multiplicity reactivation in these experiments is simply to permit the observation of progeny from phage which have received a high dose of irradiation.

The appearance of mutants among the survivors following ultraviolet-irradiation has been reported for a number of other phage systems. In some cases mutation has been observed after irradiation of the host cell only (T3, Fraser, 1957;  $\lambda$ , Jacob, 1954). In these cases a more pronounced effect is observed if both the phage and host cell are irradiated (Weigle and Dulbecco, 1953; Weigle, 1953). A possible increase in mutation rate following irradiation of the free phage alone has been reported for T1 (Tessman, 1956). In

all these cases, the possibility of homology between the phage and host cell renders ambiguous the origin of the observed mutations. It seems most probable that many of the mutations result from genetic recombination between the phage and a non-wild-type portion of the phage homolog carried by the host cell.

## VI. SUMMARY AND PRINCIPAL CONCLUSIONS

The inactivation by radiation of T-even bacteriophage is primarily a result of discrete lesions produced in the genetic structure of the particle. These lesions have effects in several types of experiments which are closely restricted to the region around the hit. Thus, in a genetic cross between inactive and nonirradiated phage, a genetic marker which happens to be remote from any lesion will appear in the progeny. A marker which happens to be close to a lesion is less likely to do so. The probability of such a marker rescue is like the probability of ordinary genetic exchange in that it depends on distance. The biological function of a hit region of the genome is inactivated by the hit. Other unhit regions in the same particle retain their functions. These conclusions provide a basis for understanding the two oldest and most provocative experiments in phage radiobiology—multiplicity reactivation and the Luria-Latarjet Experiment.

Multiplicity reactivation occurs whenever an injected cell contains at least one complete unhit set of genes whose function is necessary for the process of duplication and recombination between genomes. Recombination between particles then occurs with a sufficiently high efficiency to assure in most cases the reconstitution of an undamaged genome.

The Luria-Latarjet effect is to be understood in similar terms. During the first 6 minutes after infection the preduplicational gene functions are being performed. The onset of duplication at 6 minutes creates essential immunity to radiation, since multiplicity reactivation is limited then only by the degree to which recombination between damaged genomes falls short of being perfectly efficient.

The picture as presented is at present primarily a formal one. It will achieve more meaning upon the identification of the genes (cistrons) which perform preduplicational functions. It seems likely that two of these have been identified for the special case of T4 growing in K12( $\lambda$ ). One would anticipate that the gene(s) required for the synthesis of hydroxymethyl cytosine will turn out to be another.

It is likely that the radiobiology of all phages cannot be forced into this picture. It is equally likely that the experiments and ideas applied to T-even radiobiology will supply guidance for future studies with other systems.



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